Discovering Novel Feedback and Crosstalk Mechanisms in Cellular Signaling Pathways

Discovering Novel Feedback and Crosstalk Mechanisms in Cellular Signaling Pathways

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Discovering Novel Feedback and Crosstalk Mechanisms in Cellular Signaling Pathways

Abstract

Multiple signaling pathways control cellular response to environmental cues such as nutrients, growth factors and stress. Interpretation of these cues requires coordinated regulation of intracellular signaling pathways. Our attempt to understand how cells coordinate different signaling pathways led to the discovery of two crosstalk mechanisms between different signaling cascades.

We found that PI3K-AKT signaling reduces EGFR signaling to the parallel ERK-MAPK pathway by enhancing EGF induced EGFR degradation. At the molecular level AKT activates PIKfyve to facilitate EGFR trafficking from early endosomes to the lysosomes.

Using a mass spectrometry based approach we also found growth factor signaling by EGF inhibits stress response. In particular, inhibiting RSK signaling downstream of EGF increased the activity of stress activated kinases p38, MSK2 and ERK5. We propose that when growth factors are present active RSK phosphorylates and inhibits a master regulator of stress response MEKK3, which leads to termination of MEKK3 signaling to downstream kinases.

Our unbiased phosphoproteomic approach also lead to identification of many ERK and RSK substrates that will help us explain how growth factor signaling regulates a wide variety of biological processes.

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And final thanks go to God. As in “Thank God it is almost over.”

CHAPTER 1

INTRODUCTION

Cell Signaling Basics

Biological systems rely on proper reception and efficient interpretation of extracellular and intracellular cues to thrive. Cells monitor the availability of nutrients; energy; growth factors; connections with other cells and the extracellular matrix. Stress factors such as DNA damage, hyper osmolarity and inflammation are also monitored by the cell to make adaptive changes. In response to these changes cells decide to grow, proliferate, migrate, differentiate, change their metabolism or undergo cell death (Figure 1.1).

Misperception or misinterpretation of extracellular and intracellular signals leads to disruption of cellular and organismal homeostasis and can be detrimental. Therefore, living organisms employ intricate signaling pathways to ensure proper regulation of biological processes by regulating the functions of each protein in the cell. Such regulation occurs at the transcriptional and translational level to regulate protein abundance but also happens at the post-translational level where protein function as well as its abundance is regulated.

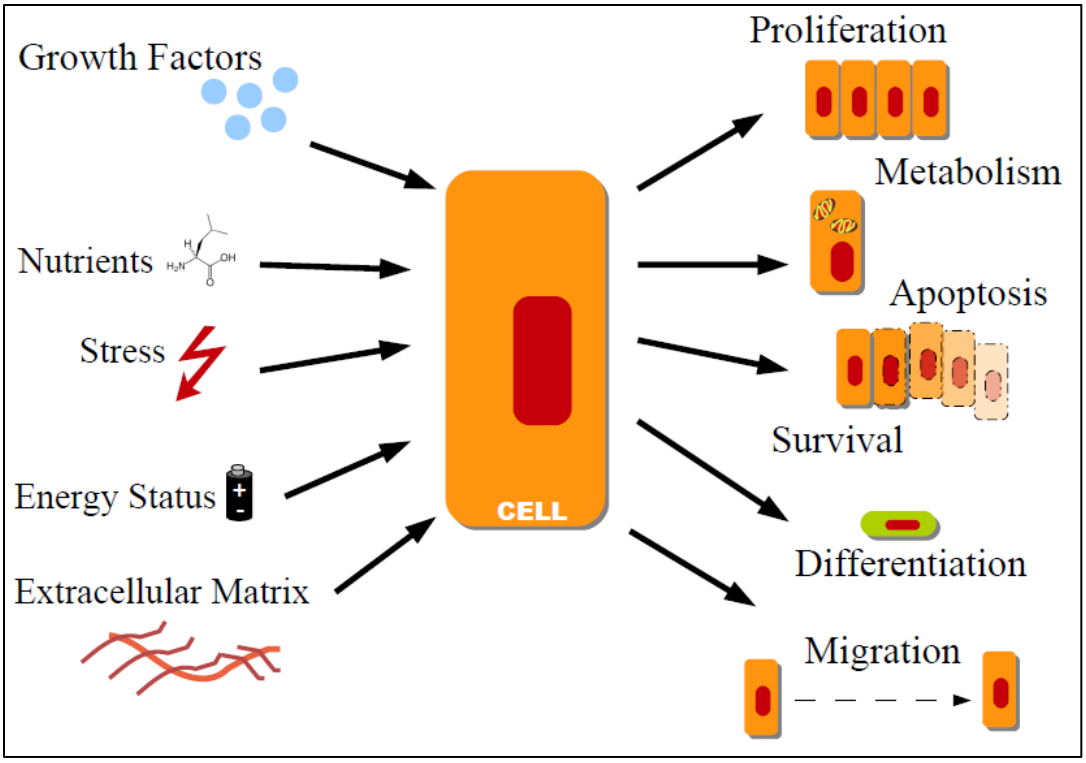

Figure 1.1 Cells interpret environmental cues to regulate biological processes

One mechanism of post-translational regulation is reversible protein phosphorylation. Kinases transfer the gamma-phosphate group of adenosine triphosphate (ATP) to serine, threonine and tyrosine residues on substrate proteins (Berg et al., 2007). Substrate proteins can also be de-phosphorylated by phosphatases. The sequence surrounding the serine, threonine and tyrosine residues (the target motif) enable kinases to specifically phosphorylate substrates. Substrate specificity is also provided by docking and interaction domains, scaffold proteins and subcellular localization. A protein's phosphorylation status can alter its conformation, its association with other proteins, determine its stability and ultimately regulate its function. Because of their ability to be dynamically and specifically regulated, living organisms utilize protein phosphorylation events as "cascades" to transmit information throughout the cell.

In this work I will focus on three of the cells major signaling cascades: 1) extracellular signaling regulated kinase – mitogen activated protein kinase (ERK-MAPK) signaling cascade which is involved in cell proliferation, survival and migration, 2) p38-MAPK cascade which is involved in stress signaling to regulate cell survival, cell death and inflammatory response and 3) phosphatidylinositol-3 kinase-AKT-mechanistic target of rapamycin complex 1 kinase (PI3K-AKT-mTORC1) pathway which is a master regulator of cell metabolism and growth. These signaling pathways can be activated by extracellular matrix, growth factors and cytokines, which bind to cell surface receptors. Receptor activation leads to recruitment of signal intermediate proteins that activate a cascade of kinases, which lead to substrate phosphorylation and a biological output (Figure 1.2).

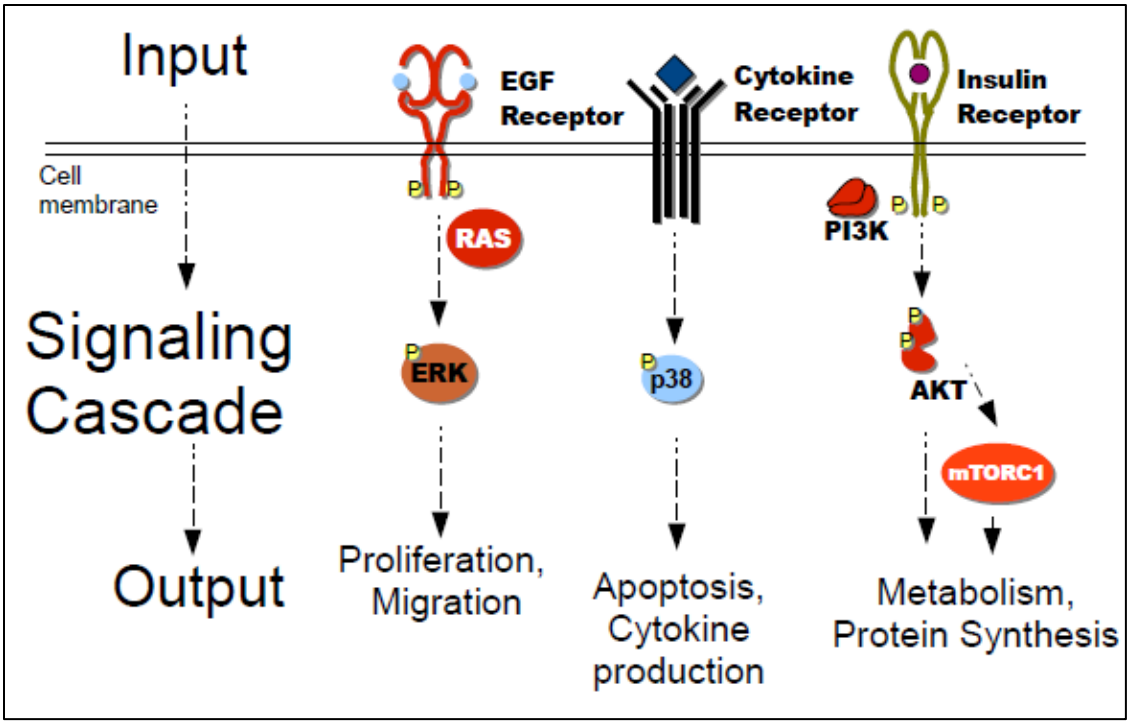

Figure 1.2 Cellular signaling can be initiated by cell surface receptors. These receptors activate a cascade of kinases that transmits extracellular cues to downstream effector proteins to regulate biological processes. Intermediate kinases are omitted for clarity. Yellow circles represent phosphorylation events. See figures 1.4, 1.5 and 1.6 for details of ERK, p38 and AKT signaling cascades.

Even though signaling cascades were initially thought to be activated and regulated by distinct environmental cues to regulate separate biological processes, work over the past two decades has shown that cellular signaling cascades extensively communicate with each. The Blenis laboratory is interested in answering two fundamental cell biological questions: How are different signaling pathways regulated in a coordinated fashion and what are the targets of signaling pathways that ultimately regulate biological processes such as cell proliferation, growth, migration, survival and cell death? This dissertation describes two newly identified mechanisms of pathway crosstalk. In Chapter 2, I will discuss how AKT regulates ERK-MAPK pathway by regulating EGFR degradation. In Chapter 3, I will provide evidence for inhibition of p38-MAPK stress pathway by ERK-MAPK signaling and describe the mechanistic link between these two pathways. In Chapter 4, I will give examples of newly identified substrates of ERK-MAPK signaling pathway which help us understand how ERK signaling regulates biological processes such as migration, proliferation and survival.

Epidermal Growth Factor Receptor Signaling

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK) that is essential for normal development (Sibilia et al., 2007). EGFR belongs to the EGFR family of RTKs that include ErbB2, ErbB3 and ErbB4. In the absence of their ligand, EGFR molecules form dimers and dissociate from each other at a constant rate. Ligand binding stabilizes the EGFR dimers (Chung et al., 2010). EGFR ligands include EGF, heparin binding EGF (HB-EGF), betacellulin (BTC), transforming growth factor alpha (TGF-alpha), amphiregulin (AR), and epiregulin (EREG) (Yarden and Sliwkowski, 2001). EGF, HB-EGF and TGF-alpha specifically bind EGFR with high affinity whereas BTC can also bind ErbB4 with high affinity

(Jones et al., 1999). In addition to stabilizing homodimers of EGFR, ligand binding also causes receptor heterodimerization of EGFR with ErbB2, ErbB3 and ErbB4.

Once stabilized EGFRs phosphorylate each other on multiple tyrosine residues. Phosphorylated residues on EGFR create docking sites for downstream signaling adaptors and scaffold proteins. For example, upon activation, EGFR activates phospholipase-C gamma (PLC-gamma) through PLC-gamma Src homology 2 (SH2) domain binding to EGFR phosphorylated at Tyr992 (pTyr992), which activates the downstream protein kinase C signaling (PKC) (Emlet et al., 1997) (Singer et al., 1997). EGFR associates with growth factor receptor bound protein 2 (GRB2) via EGFR pTyr1068 binding SH2 domains on Grb2. EGFR-GRB2 association activates the small guanosine triphosphate hydrolyzing enzyme (GTPase) RAS, which initiates the ERK-MAPK signaling cascade (Mendoza et al., 2011a). GRB2 also recruits GRB2 associated binder 1 (GAB1). In turn, GAB1 brings the PI3K regulatory subunit p85 to the plasma membrane, which results in activation of the PI3K-AKT signaling cascade. Through Tyrosine-X-X-Methionine motifs, where X represents any amino acid, ErbB3 directly binds p85 in the context of EGFR-ErbB3 heterodimers and activates PI3K (Hellyer et al., 2001). In addition to autophosphorylation of the receptors, active EGFR phosphorylates downstream substrates such as Vav2 and EGFR substrate 8 (EPS8) (Duan et al., 2011) (Fazioli et al., 1993). Vav2 and EPS8 stimulate RAC1 GTPase activity (Duan et al., 2011; Scita et al., 1999). On the other hand, Tyrosine 1045 phosphorylation recruits the Casitas B-lineage Lymphoma (Cbl) E3 ubiquitin ligase which conjugates single ubiquitin residues on multiple lysines on EGFR that target the receptor for trafficking to the lysosomal compartment where it gets degraded (Sorkin and Goh, 2009). PTP1B phosphatase dephosphorylates EGFR at the contact sites between the endoplasmic reticulum and the multi-vesicular bodies of the late endosomes, yet other

phosphatases may also act on phosphorylated EGFR in the cytoplasm (Eden et al., 2010) (Kleiman et al., 2011) (Figure 1.3). Dephosphorylation and degradation terminate EGFR signaling.

EGFR can activate the cell's major signaling cascades that regulate proliferation, survival, metabolism, migration and cell death. Therefore, it is not surprising that the loss of EGFR function in the developing organism can be lethal. Knockout of the *EGFR* gene in mouse causes a plethora of developmental defects depending on the mouse strain. The most pronounced result of EGFR knockout is embryonic lethality and the survivors show epithelial defects in skin, hair and eyes as well as defects in heart, bone, brain and lung function (reviewed in (Sibilia et al., 2007)). Singly knocking out each of the EGFR ligands does not have as adverse effects possibly due to compensation from other ligands. On the other hand, triple EGF, TGFalpha and AR knock-out causes abnormalities in skin, hair, eye and mammary gland functions, (reviewed in (Sibilia et al., 2007)), double BTC and HB-EGF null mice have shortened lifespan associated with heart failure (Jackson et al., 2003). Despite the ligands ability to compensate for each other loss, over-expressing each ligand has shown non-overlapping functions. For example, EGF over-expression causes infertility and growth retardation, BTC transgenic mice has a high rate of post natal mortality with reduced body size (Schneider et al., 2005) and TGF-alpha over-expression causes epithelial hyperplasias of the mammary gland and liver (reviewed in (Sibilia et al., 2007)).

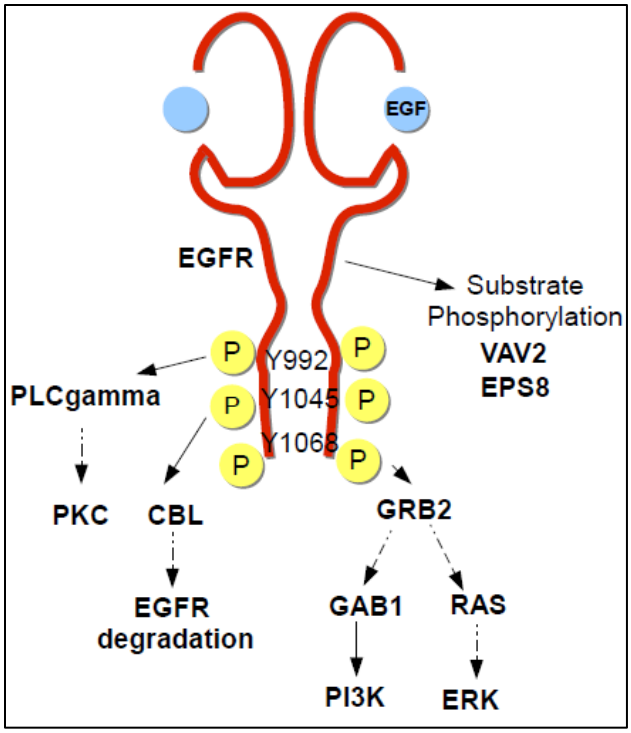

Figure 1.3 Simplified representation of EGFR regulating multiple signaling pathways.

In addition to regulation of proper development, EGFR signaling is also involved in pathogenesis of variety of human cancers. EGFR over-expression is common in many forms of human cancers (Roberts and Der, 2007). 30% of the breast cancers over-express ErbB2, which is shown to up-regulate EGFR stability and function in 3-dimensional mamosphere models as well as in suspension (Grassian et al., 2011). EGFR mutations are also a common driver event in tumorigenesis: 10% of non-small cell lung cancers and 20% of glioblastomas have hyperactive EGFR signaling due to EGFR mutations. L834R mutation and deletion of exons 2 through 7 (EGFR vIII) are among mutations that hyper-activate EGFR signaling (Riese et al., 2007). Pharmacological inhibition of the EGFR kinase causes cell death in many tumor cell lines that have hyperactive EGFR signaling, highlighting the addiction of these cells to EGFR signaling for cell survival. Furthermore, a commonly observed mechanism of resistance to ATP competitive EGFR inhibitors is the EGFR T790M mutation. This mutation causes structural change in the inhibitor-binding region of EGFR. While the mutant EGFR can still bind ATP, it can no longer bind the inhibitor and therefore can resume downstream signaling even in the presence of the inhibitor.

ERK-MAPK Signaling Cascade

ERK is a serine-threonine kinase that phosphorylates serine or threonine residues of substrates within the target motif Pro-X-Ser/Thr-Pro where X is any amino acid. Two isoforms of ERK, ERK1 and ERK2 share 84% amino acid sequence homology. ERK is activated by G-protein coupled receptors, integrins and growth factors (Roberts and Der, 2007). In context of EGFR signaling, following ligand engagement, GRB2 binds directly to EGFR on pTyr1068 or through binding to Src homology domain containing transforming protein 1 (SHC1) which binds to EGFR on pTyr1173. GRB2-EGFR interaction leads to membrane recruitment of guanine

nucleotide exchange factor (GEF) for the RAS GTPase: Son of Sevenless (SOS). SOS-RAS interaction loads RAS with GTP and activates it. GRB2 also binds GAB1. Tyrosine phosphorylated GAB1 binds the RAS GTPase activating proteins (GAPs). RAS-GAP proteins such as p120 and Neurofibromin 1 (NF1) enhance GTP hydrolysis by RAS, which inactivates RAS. GAB1 is dephosphorylated by the SH2 domain containing protein tyrosine phosphatase (SHP2). GAB1 dephosphorylated on its tyrosine residues has a lower affinity for p120 and therefore SHP2 mediated GAB1 dephosphorylation ensures continued RAS signaling. Active RAS then recruits RAF isoforms, A-RAF, B-RAF and C-RAF to the plasma membrane for activation. RAS binding induces RAF heterodimerization and protein phosphatase 2A (PP2A) mediated dephosphorylation of C-terminal serine residues, which bind the inhibitory 14-3-3 proteins. Through membrane recruitment dephosphorylation-phosphorylation cycles, hetero and homo-dimerizations, RAF proteins are released from their auto-inhibited state (McKay and Morrison, 2007). RAF kinases are MAPK kinase kinases (MAPKKK) and have very narrow substrate specificity: they phosphorylate and activate the MAPK kinases MEK1 and MEK2 (MAPKKK). MEK is a dual specificity kinase that phosphorylates Tyr and Thr residues on ERK1-2 activation loops. The only known substrate of MEK is ERK. Having narrow substrate specificity kinases such as RAF and MEK allows for signal amplification and provides regulatory nodes for the signaling cascade, which will be described in detail in section "Temporal Regulation of Signaling Pathways". Active ERK phosphorylates a variety of substrates including transcription factors, cytoskeletal elements and structural proteins and effector kinases such as p90 ribosomal S6 kinase (RSK) and mitogen and stress activated kinase (MSK) (Yoon and Seger, 2006) (Figure 1.4).

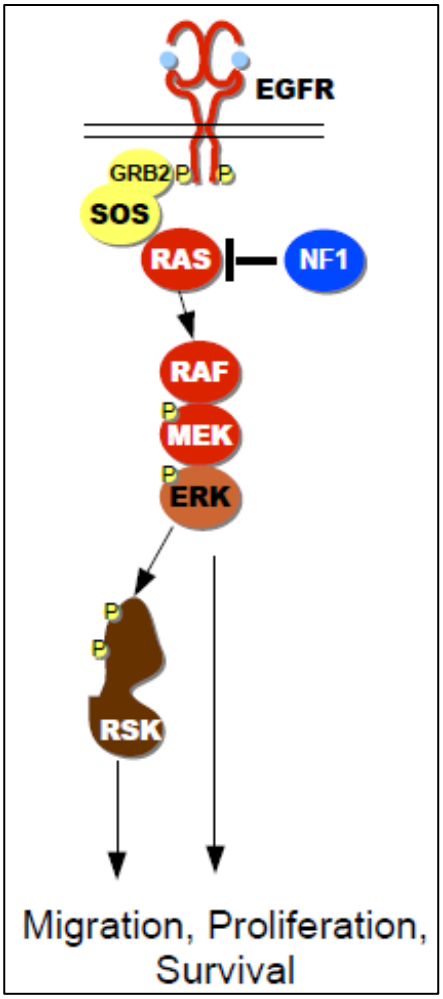

Figure 1.4 EGFR initiates the ERK-MAPK signaling by activating RAS small GTPase, which activates the RAF-MEK-ERK kinase cascade. Active ERK phosphorylates effector proteins some of which are also kinases, such as p90 RSK.

RSK and MSK are members of the AGC family of kinases, which are named after protein kinase A (PKA) protein kinase G (PKG) protein kinase C (PKC). This family also includes S6K1-2, AKT1-3, serum and glucocorticoid regulated kinase SGK1-3. These kinases phosphorylate their substrates on basophilic amino acid sequences with the target motif Arg-X-Arg-X-X-Ser/Thr (Pearce et al., 2010). Arg residue 5 amino acids upstream (-5 position) of the phosphorylated serine and threonine can be substituted with Lys for RSK, MSK and S6K, however AKT has a stricter requirement for this -5 Arg at the target motif. Among the other AGC kinase family members RSK and MSK are unique in that they have two catalytically active kinase domains. The N-terminal kinase domain (NTKD) belongs to the AGC family whereas the C-terminal kinase domain (CTKD) belongs to the CamK family of kinases. ERK phosphorylation of RSK Thr573 and MSK Thr581 promotes activation of their CTKD. p38 MAPK also phosphorylates and activates MSK in response to cellular stress. On the other hand, RSK is activated by p38 only in dendritic cells via a p38 mediated MAPK activated protein kinase 2 and 3 (MK2-3) (Zaru et al., 2007). Once activated the CTKD of MSK autophosphorylate Ser376, Ser381 in the linker region and the NTKD activation loop Ser212. CTKD of RSK S380 and create a docking site for phosphoinositide dependent kinase (PDK1). The activation loop of the NTKD of RSK is phosphorylated on Ser221 by PDK1 and is activated. Once active, NTKDs of RSK and MSK phosphorylate substrate proteins. MSKs reside constitutively in the nucleus. ERK and RSK phosphorylate cytoplasmic proteins and they translocate to the nucleus within minutes of activation (Chen et al., 1992) where together with MSKs target transcription factors involved in a variety of biological processes.

ERK signaling is critical in development and deregulation has been implicated in a variety of human pathologies. Deletion of the K-Ras in mice is embryonic lethal whereas H-Ras

or N-Ras deletion has no obvious phenotypes suggesting at the developmental stages there may be redundancy between different Ras isoforms (Johnson et al., 1997). B-Raf and C-Raf deletion in mice is also embryonic lethal. A-Raf deletion show neurological and intestinal disorders (Baccarini, 2005). Similar to B-Raf, C-Raf and K-Ras deletion, Mek1 and Erk2 deletion in mice is also embryonic lethal whereas Mek2 and Erk1 null mice are viable with Erk1 null mice showing defects in the immune system and adipocyte differentiation (Aouadi et al., 2006; Belanger et al., 2003; Bissonauth et al., 2006). RSK2 null mice have cognitive deficiencies as well as defects in white adipose tissue, whereas RSK1 and RSK3 null mice have no obvious phenotypes. In stark contrast to other members of the ERK signaling cascade, MSK1 and MSK2 compound deletions and RSK1-RSK2-RSK3 compound deletions in mice have no obvious developmental defects, are viable and fertile suggesting there is a great deal of redundancy at this level of the MAPK signaling cascade (Cargnello and Roux, 2011). Deregulation of this pathway is involved in both developmental disorders as well as human malignancies. RASopathies are a class of human disorders in individuals with mutations in the RAS-MAPK signaling pathway. Mutations and deletions occur at the *PTPN11* gene (encodes SHP2), *KRAS, SOS1, RAF1, MAP2K1-2* (encode MEK1-2), *RASA1* (encodes p120 RASGAP) and *NF1* (encodes the RAS-GAP NF1) (Tidyman and Rauen, 2009). RAS mutations are among the most frequent events in human cancers. RAF mutations are very common in human melanomas with the BRAF V600E being the most common. MEK1 is also mutated as a resistance mechanism to RAF inhibition for reactivating ERK signaling (Emery et al., 2009). ERK2 is also recently found to be amplified in cell culture models of drug resistance to EGFR inhibitors (Ercan et al., 2012).

p38 MAPK Signaling Cascade

p38 MAPK signaling cascade is the cell's major stress response pathway. p38 gets activated by cytokines, pathogens, environmental stress such as osmotic stress and reactive oxygen species (Cuadrado and Nebreda, 2010). Just as in the ERK MAPK signaling cascade, p38 signaling cascade is initiated by MAPKKKs, including Apoptosis signal regulating kinase (ASK1), transforming growth factor beta (TGF-beta) activated kinase (TAK1) and MEKK3. Oxidative stress causes thioredoxin oxidation on its cysteine which frees ASK1 for autophosphorylation and activation (Matsukawa et al., 2004). TGF-beta stimulation causes tumor necrosis factor (TNFalpha) receptor associated factor 6 (TRAF6) E3 ubiquitin ligase association with the TAK1 binding protein 1 (TAB1). TRAF6 auto ubiquitination drives binding of TAB1: an event leading to activation of TAK1 through a mechanism that possibly involves oligomerization. Osmotic stress causes osmosensing scaffold for MEKK3 (OSM) binding to RAC1, which brings MEKK3 to the plasma membrane (Uhlik et al., 2003). MEKK3 is activated by oligomerization and autophosphorylation, which leads to phosphorylation of the downstream MAPKKs. MKK3, MKK6 activate p38 isoforms by phosphorylating them on their activation loops whereas MKK4 and MKK7 are more established activators of the parallel MAPK pathway: the c-Jun N terminal kinase (JNK) cascade (Cargnello and Roux, 2011). MEKK3 can also hetero-oligomerize with MKK5, which leads to activation of ERK5 (Sun et al., 2001). Once activated p38 and ERK5 both phosphorylate proline directed serine and threonine residues on a variety of substrates that regulate the stress response. Like ERK1 and ERK2, p38 phosphorylates and activates MSK1 and MSK2. RSK is activated by p38 and ERK5 only in dendritic cells and downstream of hyperactived MKK5, respectively, suggesting that RSKs activation is more specific to ERK1 and ERK2 than to p38 and ERK5 (Ranganathan et al., 2006;

Zaru et al., 2007). On the other hand, MAPK activated protein kinase MK2 and MK3 are activated by p38 isoforms but not ERK1-2. p38 also activates several transcription factors involved in apoptosis and immune response (Figure 1.5).

p38 alpha knockout mice die in embryogenesis as do MKK3 and MKK6 double knockout mice suggesting a role for p38 activation during development (Cuadrado and Nebreda, 2010). However, deletion of other p38 isoforms is not lethal pointing out some redundancy during development. Also, deletion of neither MKK3 nor MKK6 is embryonic lethal, however MKK3 null mice have reduced immune response due to reduced cytokine production by the macrophages and dendritic cells (Lu et al., 1999). Similarly, MEKK3 deletion also causes reduction in cytokine production, but in addition to reduced p38 signaling, inhibition of NFkB signaling, which can also be activated downstream of MEKK3, also contributes to this effect (Yang et al., 2001). Both TAK1 and MEKK3 null mice are embryonic lethal with MEKK3 null mice showing inappropriate cardiovascular development (Tang et al., 2008; Yang et al., 2000). In contrast, ASK1 null mice are viable and fertile, however fibroblasts derived from these mice show reduced apoptosis in response to hyperosmotic shock and TNF alpha due to their inability to provide sustained p38 and JNK activation (Tobiume et al., 2001).

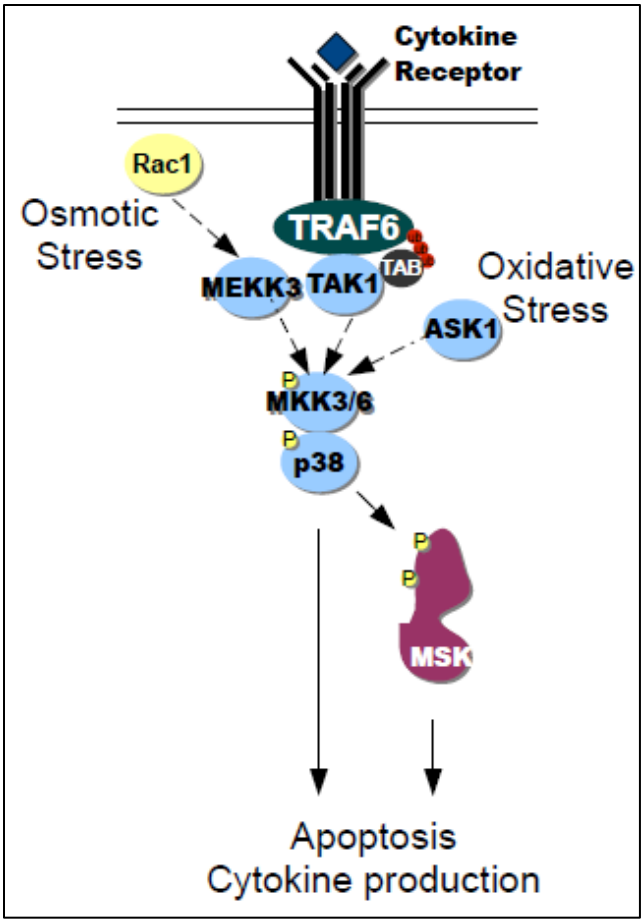

Figure 1.5 Various stress factors and cytokines activate MAPKKKs, which initiate a signaling cascade to MAPKKs to activate p38-MAPK. Only MEKK3, TAK1 and ASK1 are shown for simplicity. Red circles represent ubiquitination events. Additional MAPKKKs have also been described (Cuadrado and Nebreda, 2010).

PI3K-AKT-mTORC1-S6K Signaling

PI3K is a phosphoinositide kinase that phosphorylates the 3 position of the phosphatidylinositol. There are three classes of PI3Ks that differ in their substrate specificity and function. Class I PI3K is composed of two subunits, p85 (alpha and beta), p55, p150, p101 or p87 are the regulatory subunits and p110 (alpha, beta, gamma and delta) is the catalytic subunit. Class I PI3Ks phosphorylate the 3 position of phosphatidyl-inositol 4,5 bisphosphate ($PI(4,5)P_2$) to generate $PI(3,4,5)P_3$ whereas Class III phosphorylates only PI and Class II PI3K phosphorylate PI as well as PI(4)P (Engelman et al., 2006).

In the context of insulin signaling, the activated insulin receptor gets cross-phosphorylated on multiple tyrosine residues which recruit the insulin receptor substrate (IRS) proteins to the plasma membrane. This is analogous to GRB2 recruitment by EGFR. IRS1-2 recruit the regulatory p85 subunit of the Class I PI3K to the plasma membrane. While providing structural stability to the p110, p85 also inhibits the basal PI3K activity. RAS binding to the catalytic subunit p110 subunit further activates PI3K and this interaction is necessary for PI3K's ability to induce oncogenic transformation. A major effector of PI3K is AKT kinase (Franke et al., 1997). Interaction between the pleckstrin-homology (PH) domains on AKT and $PI(3,4,5)P_3$ recruit AKT to the plasma membrane where it is phosphorylated by PDK1 on Thr308. AKT is fully activated by Ser473 phosphorylation which is carried out by mTORC2. This complex is composed of rapamycin insensitive companion of mTOR (RICTOR), mammalian stress activated kinase interacting protein (mSIN1), mammalian lethal with Sec13 protein 8 (mLST8) and mTOR. Signaling from the RTKs to AKT is antagonized by the phosphatase and tensin homolog (PTEN) phosphatase that dephosphorylates the 3 position of the $PI(3,4,5)P_3$. AKT2

can also be activated by $PI(3,4)P_2$ that is generated by the PI5 phosphatase SHIP1-2. PI(3,4)P2 is further dephosphorylated by INPP4 to PI(3)P (Gewinner et al., 2009).

Once activated, AKT phosphorylates a variety of substrates involved in metabolism and protein synthesis leading to cell growth. In 1994 our lab discovered the first clue that mTORC1 signaling is downstream of PI3K signaling (Chung et al., 1994). Two substrates of AKT are involved in connecting PI3K-AKT signaling to mTORC1 signaling. mTORC1 is composed of the serine threonine kinase mTOR, mLST8, regulator-associated protein of mTOR (Raptor) and is bound by the inhibitory proline rich AKT substrate of 40kDa (PRAS40). Phosphorylated PRAS40 is sequestrated by the 14-3-3 proteins and can no longer inhibit mTORC1 (Vander Haar et al., 2007). AKT also phosphorylates tuberous sclerosis complex 2 (TSC2), when phosphorylated can no longer inhibit the small GTPase Ras homolog enriched in Brain (RHEB). RHEB in conjunction with RAG GTPases, which are activated downstream of the amino acid sensing pathway, activates mTORC1 complex (Sengupta et al., 2010). In contrast, low energy levels in cells lead to liver kinase B1 (LKB1) mediated activation of AMP kinase, which phosphorylates and inhibits mTORC1(Shaw, 2009). Two most established substrates of the mTORC1 complex, Eukaryotic elongation initiation factor 4E (EIF4E) binding protein (4E-BP1) and S6K, interact with Raptor via their Tor signaling (TOS) motif (Schalm and Blenis, 2002; Schalm et al., 2003). 4E-BP1 phosphorylation by mTORC1 dissociates 4E-BP1 from EIF4E which upregulates mRNA translation. mTORC1 phosphorylation of S6K on Thr389 creates a docking site for PDK1 which in turn phosphorylate the activation loop of S6K. Active S6K in turn phosphorylates a large number of substrates involved in mRNA translation, splicing and ribosome biogenesis (Figure 1.6).

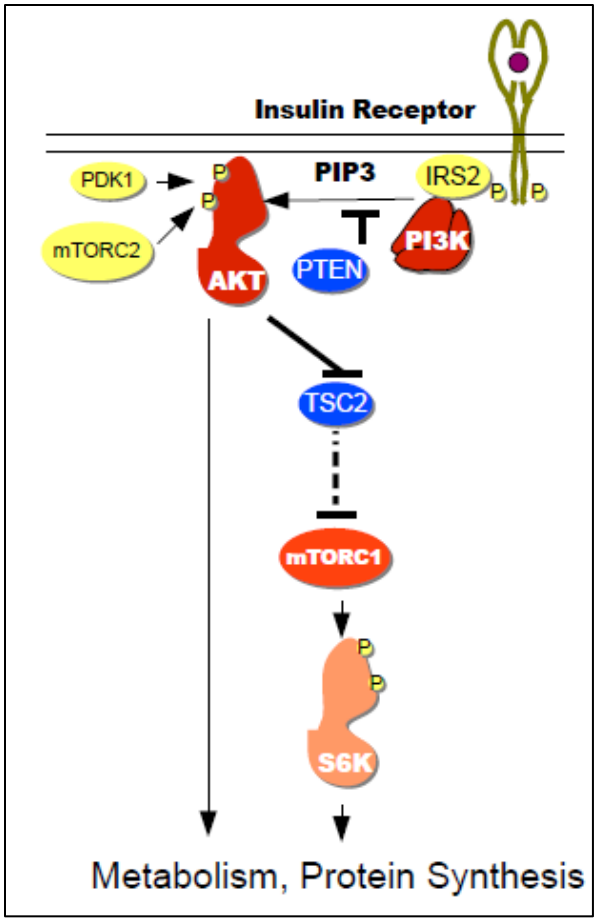

Figure 1.6 Type I PI3Ks activate AKT downstream of RTKs. Active AKT can directly regulate end point proteins and also activate mTORC1 by phosphorylating and inhibiting TSC2 and PRAS40 (not shown) both of which inhibit mTORC1 activity.

Given the fact that PI3K signaling is a multifaceted regulator of growth and metabolism it is not surprising that it is one of the most frequently mutated kinase in human cancers (Steelman et al., 2011). p110 alpha H1047R, E545K and E542K mutation hyper-activate the kinase activity leading to increased PIP3 production and AKT activation(Gymnopoulos et al., 2007). The loss of the tumor suppressor PTEN either through mutational inactivation or deletion is a common event in human cancers and is the cause of Cowden's disease which is characterized with benign tumors that can progress into malignancy (Krymskaya and Goncharova, 2009). Similarly inactivation and loss of the mTORC1 negative regulators LKB1 and TSC2 lead to Peutz-Jeghens and tuberous sclerosis syndrome, respectively, both of which are characterized by small tumors throughout out the body (Krymskaya and Goncharova, 2009). AKT1 is also mutated in variety of cancers. AKT1 E17K mutation allows AKT1 PH domain to bind PI(4,5)P2 in addition to PI(3,4,5)P3 which facilitates AKT activation (Carpten et al., 2007). Increased signaling downstream of AKT2 and AKT3 is achieved through their genomic amplification and the E17K mutations are much less common than observed for AKT1 (Steelman et al., 2011).

As increased PI3K signaling is associated with tumorigenesis, its reduction causes insulin resistance, which is part of the pathology of type 2 diabetes. A major role of PI3K signaling is to increase hepatic glucose uptake in response to increasing levels of insulin in the plasma. Once activated AKT phosphorylates the GAP for Rab8, AKT substrate of 160 (AS160) (Sano et al., 2003). Phosphorylation inhibits AS160 and therefore allows Rab8 function, which leads to localization of glucose transporter 4 (GLUT4) from the intracellular vesicles to cell surface. This allows for glucose uptake by the cells. AKT also stimulates glycogen synthesis by phosphorylating and inhibiting glycogen synthase kinase 3 (GSK3).

Temporal Regulation of Signaling Pathways

The importance of the ERK, AKT, p38 signaling cascades is underlined by the observation that loss of function of function of members of these pathways result in systemic defects during development. On the other hand, hyper-activation of these pathways are involved in various human disorders and cancers. Therefore, organisms tightly regulate the activity level of these pathways, through multiple mechanisms that involve pathway feedback, crosstalk as well as compensation (Mendoza et al., 2011a).

1) Feedback

The linear nature of the RAS-RAF-MEK-ERK cascade provides a platform for amplifying the mitogenic signals. However, without the proper regulation of this platform, small changes in the input signal, such as EGF concentration, would lead to very large fluctuations in the output ERK activity. The magnitude and duration of the ERK activity output are important in cellular decision making. For example, nerve growth factor (NGF) stimulation of the PC12 cells results in a high and sustained ERK activity, which leads to differentiation. On the other hand, EGF stimulation causes a transient ERK activity which leads to proliferation (Murphy and Blenis, 2006). The ability of NGF to stimulate stronger and sustained ERK activity has been attributed to the observation that NGF receptors are not degraded following stimulation, whereas EGF stimulation of EGFR causes receptor internalization and degradation in the lysosomes.

Feedback termination of the EGFR signaling is mediated by EGF induced EGFR degradation (von Zastrow and Sorkin, 2007). As mentioned above, activated and tyrosine phosphorylated EGFR recruits multiple adaptor and scaffold proteins to initiate signaling cascades. Active EGFR also recruits components of the endocytic machinery which promote EGFR

internalization into the cells. EGFR activity is required for the internalization processes since, EGFR catalytic inhibitors or expression of EGFR mutants that lack activity, preclude proper internalization. Internalized EGFR localizes to the early endosomes marked by the early endosomal antigen 1 (EEA.1), which is a RAB5 effector protein. From EEA.1 endosomes EGFR continues to signal and associate with GRB2 (Di Guglielmo et al., 1994). Continued EGFR activity and monoubiquitination target it to the multi-vesicular bodies (MVBs). Monoubiquitins are recognized by endosomal sorting complex for transport (ESCRT) proteins and EGFR is sorted into the lumen of the MVBs where it is separated from the signaling proteins. Once in the MVBs EGFR is destined to fuse with the lysosomes and the EGFR signal is terminated (Figure 1.7).

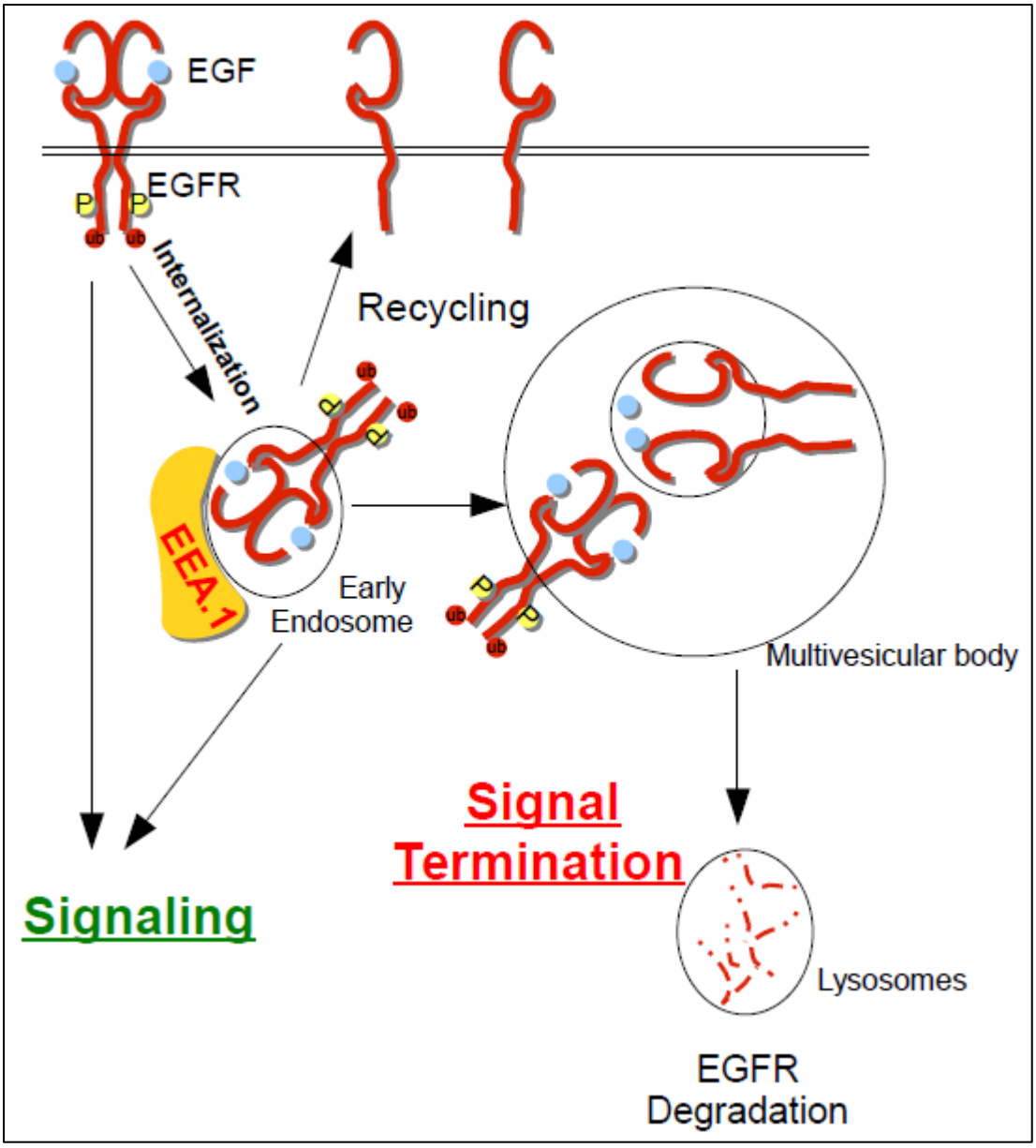

Figure 1.7 Regulation of EGFR signaling by endocytic trafficking. EGFR can signal from the plasma membrane and the early endosomes. Once sorted into the lumen of the MVB's, EGFR is separated from signaling proteins and is destined for degradation, which causes signal termination.

ERK itself is a major regulator of the upstream components of the MAPK signaling pathway. ERK phosphorylates SOS, RAF and MEK1. RSK activated by ERK phosphorylates GAB2 and SOS. These data suggest that ERK feedbacks to all levels of the RAS-ERK cascade. ERK phosphorylation of RAF reduces RAF activity by reducing RAF-RAS interaction (Ritt et al., 2010). When ERK phosphorylation sites on RAF are mutated to non-phosphorylatable Ala RAF can longer be inhibited by ERK. In a population of cells, response to increasing doses of EGF results in a graded increase in ERK phosphorylation. However, when ERK signaling to RAF is inhibited, ERK activation adapts a switch-like response to increasing stimulus (Sturm et al., 2010). This means that in response to small amounts of EGF the majority of cells do not activate ERK whereas a few cells activate ERK to the maximum extend. As EGF concentration increases, the number of cells with maximum ERK activity increases. This demonstrates how signaling feedback is required to prevent drastic fluctuation in the signaling output in response to small changes in the input. ERK also feedback phosphorylates MEK1 which reduces the activity of MEK1-MEK2 heterodimers. Mutation of this phosphorylation site on MEK1 increases ERK mediated fibroblast migration (Catalanotti et al., 2009). Additionally, ERK activates RSK which phosphorylates of GAB2 on basophilic residues leading to reduced SHP2-GAB2 interaction (Zhang et al., 2013). Because GAB1 is dephosphorylated on its tyrosine residues by SHP2, reduced GAB2 and SHP2 interaction may also increase GAB2 tyrosine phosphorylation. Similar to GAB1, tyrosine phosphorylated GAB2 may also recruit RAS-GAPs and inactivate RAS signaling. Consistently, mutation of GAB2 basophilic RSK phosphorylation sites increases ERK signaling and epithelial cell migration. Despite all having physiological consequences at the cellular level perhaps the most significant mechanism of ERK mediated feedback is ERK and RSK phosphorylation of SOS1. ERK and RSK mediated phosphorylation of SOS1 in the SOS1

proline rich region (PR) result in SOS1 dissociation from GRB2 and SOS1 binding to 14-3-3: events that prevent SOS1 activation of RAS (Corbalan-Garcia et al., 1996; Saha et al., 2012). Consistently, mutation of phosphorylation sites on SOS1 PR or deletion of this region results in hyper-activation of ERK. In fact, deletion of SOS1 PR is a naturally occurring mutation, which causes hereditary gingival fibromatosis, which is one of the RASopathies (Hart et al., 2002).

The discovery of S6K feedback to AKT signaling also carries medical implications. Hyper-activation of the mTORC1-S6K signaling axis is a common event in human cancers as judged by the high abundance of phosphorylated S6 protein in tumor samples from patient. This makes mTORC1 signaling a favorable drug target. However tumor samples collected from patients following treatment with Rapamycin display increased phosphorylation of AKT, suggesting S6K inhibition relieves the feedback inhibition of AKT (O'Reilly et al., 2006). Accordingly, in cells were mTORC1 signaling is hyper-activated either through loss of PTEN, LKB1 or TSC2, insulin stimulation does not cause AKT activation to the maximum extend (Manning, 2004). One of mechanistic basis for this observation is S6K phosphorylation of IRS1 on multiple serines, which reduces IRS1 stability and therefore disrupts signaling from the insulin or insulin like growth factor signaling to AKT. This mechanism also operates in insulin resistant mice models of Type 2 diabetes where insulin stimulation does not activate AKT, which correlates with a high level of IRS1 phosphorylation. On the other hand S6K null mice are refractory to insulin resistance and high fat induced diabetes, which parallels the lower level of IRS1 serine phosphorylation. This points out to a broken feedback loop where S6K can no longer inhibit AKT signaling and therefore AKT activation following insulin stimulation is still intact. S6K also phosphorylates RICTOR and increases 14-3-3 binding to mTORC2 complex, which reduces mTORC2 activation of AKT. Interestingly, this mechanism seems to be specific to AKT activation as

RICTOR phosphorylation does not affect SGK or PKC activation by mTORC2 (Dibble et al., 2009). In addition to S6K mediated feedback to IRS1 and RICTOR, mTORC1 complex itself phosphorylates another signaling adaptor protein GRB10. mTORC1 phosphorylation of GRB10 induces its proteasomal degradation and therefore results in reduces receptor signaling to AKT (Yu et al., 2011).

Feedback also occurs at the transcriptional level. AKT phosphorylates the Fork head transcription factor FOXO3a. Phosphorylates FOXO3a is retained in the cytoplasm by 14-3-3 binding and can no longer initiate target transcription. When AKT is inhibited, FOXO3a goes into the nucleus to initiate transcription of ErbB3 and IGF1R and enhances RTK signaling (Chandarlapaty et al., 2011). ERK regulates transcription of two classes of signaling regulators. Members of the Sprouty family of proteins act both as a positive and negative regulator of EGFR signaling. On one hand, tyrosine phosphorylated Sprouty acts a "decoy" for Cbl E3 ubiquitin ligase and prevents Cbl ubiquitination of EGFR (Rubin et al., 2003). On the other hand, Sprouty proteins also interfere with Grb2 recruitment to the RTKs (McKay and Morrison, 2007). The second class of signaling regulators is dual specificity phosphatases (DUSPs), which can dephosphorylate both Ser-Thr and Tyr residues. DUSPs associate with ERK and other MAPKs such as JNK and p38. DUSPs can regulate MAPKs by two distinct mechanisms. First is the direct dephosphorylation of the MAPKs at their activation loop phosphorylation. Nuclear DUSPs also anchor MAPKs in the nucleus where they can no longer be phosphorylated by the MAPKKs, which are exclusively cytoplasmic (Caunt et al., 2008). p38 MAPK activity also increases DUSP transcription by enhancing ATF2 transcriptional activity (Breitwieser et al., 2007). MSK1-2 activation downstream of p38 also increases DUSP1 and interleukin 10 transcription, the latter of which inhibits p38 through autocrine signaling however the

mechanism is unidentified (Ananieva et al., 2008; Mackenzie et al., 2013). Judging by the extensive feedback regulation of the AKT and ERK signaling, one would hypothesize that the parallel p38 signaling is also regulated by multiple feedback loops (Figure 1.8). One such example is the p38 phosphorylation of TAB1 (Cheung et al., 2003). Mutation of the p38 phosphorylation sites on TAB1 increases TAK1 activity towards MKK6 suggesting p38 reduces its own activity by inhibiting TAK1 activation (Singhirunnusorn et al., 2005). MK2 and MK3 activity are also required for TAB3 phosphorylation; however, whether this phosphorylation plays a role in feedback signaling is not known (Mendoza et al., 2008).

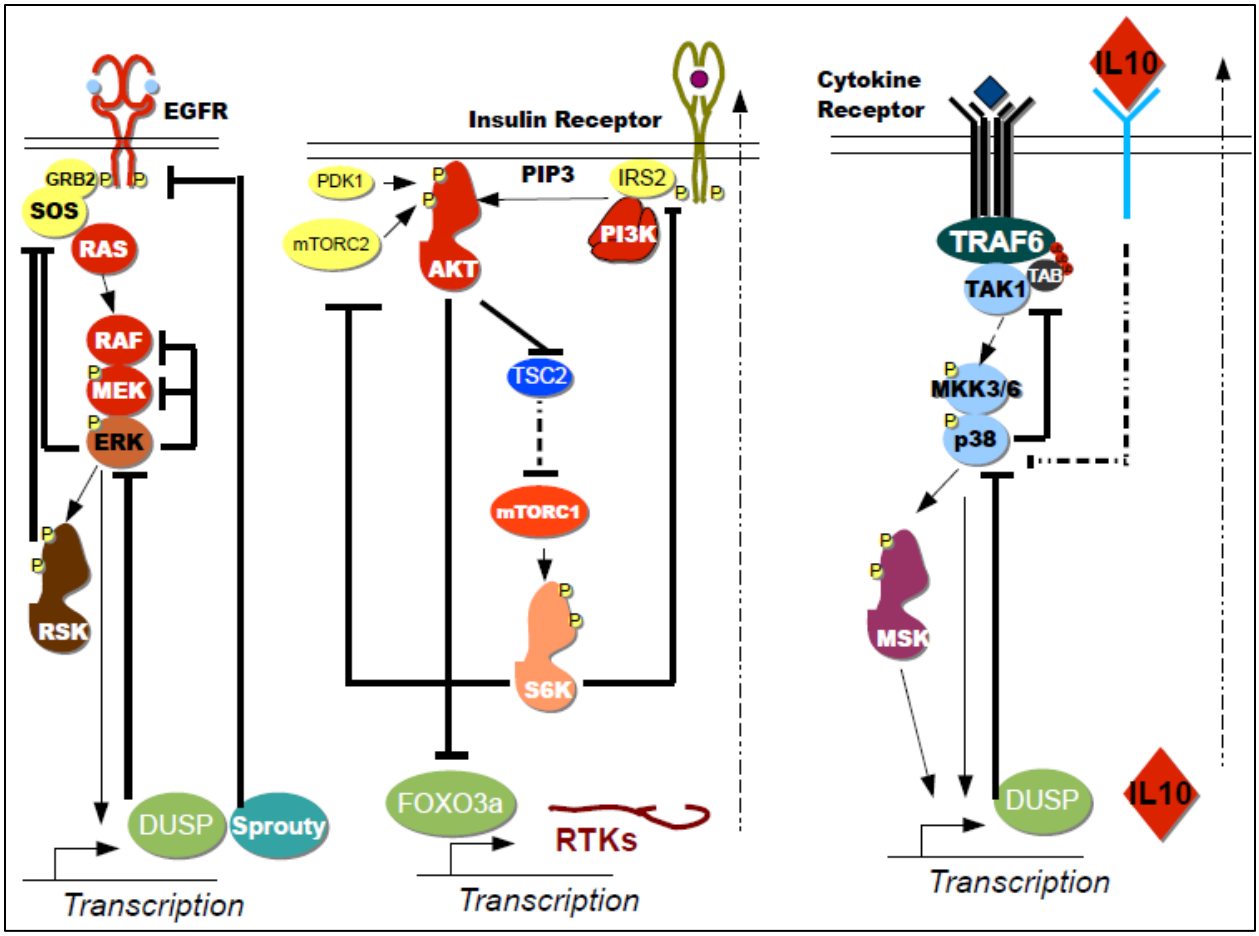

Figure 1.8 Feedback regulation of ERK, PI3K, p38 signaling pathways. Downstream kinases can either directly phosphorylate and inhibit upstream signaling proteins or initiate transcription responses that inhibit these signaling cascades via various mechanisms.

2) Crosstalk

Despite being viewed as linear signaling cascades even the earlier studies using pathway activation with distinct agonists or chemical or genetic inactivation of the certain members of these pathways suggested the amount of extensive "crosstalk" between these pathways. For example, PI3K-AKT inhibition increases ERK signaling (Rommel et al., 1999; Zimmermann and Moelling, 1999). Accordingly, AKT1 overexpression reduces ERK signaling (Irie et al., 2005). The underlying mechanism is thought to be through AKT phosphorylation of on Ser259 C-RAF which leads to 14-3-3 binding and sequestration of C-RAF (Rommel et al., 1997). The same site is also known to be basally highly phosphorylated by another member of the AGC family of kinases PKA (Dhillon et al., 2002). AKT can also phosphorylate and inhibit B-RAF on Ser428 and Ser364 (Guan et al., 2000). Mutation of Ser364 decreases 14-3-3 binding and increases B-RAF heterodimerization with C-RAF, whereas the mechanism by which Ser428 phosphorylation inhibits B-RAF activity is not clear (Ritt et al., 2010). However, AKT inhibition of B-RAF translates into reduced ERK activity only in the context of melanoma cells that carry the B-RAF activating mutation V600E (Cheung et al., 2008). This observation suggests that additional mechanisms of AKT mediated ERK inhibition may exist in normal cells. In addition to RAF isoforms, AKT also phosphorylates and inhibits ASK1 MAPKKK upstream of p38 and JNK to reduce chemotherapy induce apoptosis (Kim et al., 2001; Yuan et al., 2003). AKT regulation of JNK may also be through regulation of GTP binding to RAC1 which is reduced upon RAC1 phosphorylation by AKT on Ser71 (Kwon et al., 2000). On the other hand, AKT activation is regulated by ERK, which phosphorylates GAB1 on multiple serine residues. Inhibition of ERK increases GAB1-PI3K interaction (Yu et al., 2002). Accordingly when these sites are mutated hyperactive MEK signaling can no longer inhibit AKT activation (Lehr et al., 2004). p38

crosstalk to EGFR, ERK and mTORC1 also exists. p38 phosphorylates and recruits RAB5 effectors EEA1 and Rabenosyn-5 to the plasma membrane(Zwang and Yarden, 2006). EGFR phosphorylation on its C-terminal domain along with activation of EEA.1 and Rabenosyn facilitates ligand independent EGFR internalization. As opposed to the EGF induced EGFR internalization, p38 mediated EGFR internalization does not activate EGFR. p38 activity also enhances ERK1-2 and MEK1-2 binding to protein phosphatase 2A (PP2A), which dephosphorylates and inactivates the MEK1-2 (Junttila et al., 2008). Additionally, p38 activates p38beta activated protein kinase (PRAK), which phosphorylates and inhibits RHEB and therefore inhibits mTORC1 activity during energy stress (Zheng et al., 2011).

Crosstalk between signaling pathways is not always inhibitory. As mentioned previously RAS binding to p110 catalytic subunit allosterically activates PI3Ks. ERK pathway also crosstalks to mTORC1 signaling. Upon activation by expression of hyperactive RAS or stimulation with EGF and phorbol esters, ERK and RSK phosphorylate TSC2 and RAPTOR (Carriere et al., 2008; Carriere et al., 2011; Ma et al., 2005; Roux et al., 2004). TSC2 phosphorylation relieves the inhibition on mTORC1 and S6K, however the mechanism is not clear. RAPTOR phosphorylation by ERK and RSK stimulate mTORC1 kinase activity *in vitro* (Carriere et al., 2008; Carriere et al., 2011). RAPTOR is also phosphorylated by p38 in response to arsenide, but not to insulin suggesting that p38 can activate mTORC1 signaling in a context dependent manner (Wu et al., 2011). p38 also up-regulates mTORC1 in control of cell size, however, whether RAPTOR phosphorylation contributes to this process is not known(Cully et al., 2010) (Figure 1.9).

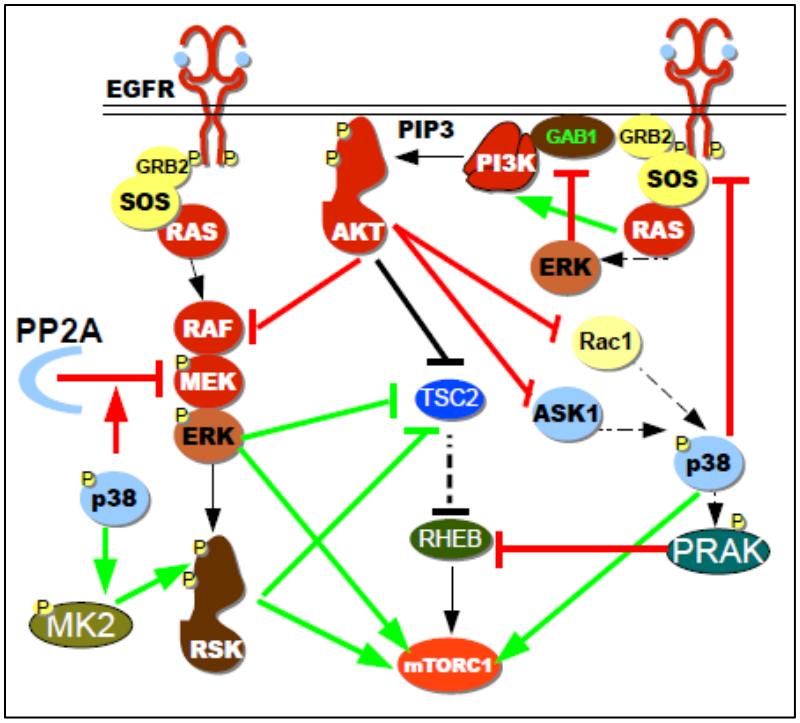

Figure 1.9 Crosstalk between ERK, PI3K and p38 pathways. These signaling interactions are detailed in the text and are often times cell type and context dependent.

Crosstalk between PI3K-AKT, ERK and p38 pathways also takes place by means of these pathways acting on the same set of substrates. For example, RSK and S6K both phosphorylate the estrogen receptor (ER) on Ser167 which enhances estrogen independent ER transactivation and ER driven transcription (Yamnik and Holz, 2010). Ribosomal protein S6 (rpS6) is phosphorylated on Ser235 and Ser236 by both RSK and S6K although Ser240 and Ser244 are exclusively phosphorylated by S6K (Roux et al., 2007). Phosphorylation enhances rpS6 binding to the 5' methyl guanosine cap of mRNAs and may enhance protein translation. In regulation of mRNA translation, p38, S6K and RSK all regulate eukaryotic elongation factor 2 kinase (eEF2K). Phosphorylation of eEF2K on Ser359 by p38 is necessary for inhibiting its activity *in vitro* (Knebel et al., 2002). Phosphorylation of eEF2K by RSK and S6K on Ser366 also inhibits eEF2K which no longer can phosphorylate and inhibit eEF2. Thus RSK and S6K enhance translation elongation by allowing eEF2 function (Wang et al., 2001).

In regulation of cell proliferation AKT and RSK both phosphorylate the cyclin dependent kinase inhibitor p27 kip1. Thr198 phosphorylation by RSK induces 14-3-3 binding and anchoring in of p27 kip1 where it inhibits RhoA and facilitates cell motility (Larrea et al., 2009). AKT phosphorylation of p27 kip1 on Ser157 also reduces p27 kip1 nuclear localization by disrupting its association with importin alpha (Shin et al., 2005b). AKT, RSK and ERK also phosphorylate and inhibit a master regulator of cell proliferation and metabolism, glycogen synthase kinase 3 (GSK3). ERK phosphorylation of GSK3alpha on Thr43 primes RSK to phosphorylate GSKalpha on Ser9. In the context of insulin signaling, AKT is the major contributor to Ser9 phosphorylation. However, in TSC2 null cells, where hyperactive S6K mediates feedback inhibition of AKT, S6K phosphorylates and inhibits GSK3 (Zhang et al., 2006b). Similarly the BCL2 associated agonist of cell death (BAD) protein is subject to

coordinated regulation by AKT, S6K and RSK. AKT and S6K phosphorylate BAD on Ser136 whereas RSK phosphorylates it on Ser112. These phosphorylation events induce BAD binding to 14-3-3, which enables the pro-survival protein BCL2 to inhibit mitochondrial apoptosis (She et al., 2005). Depending on the cell type and extracellular stimuli extensive functional redundancy between the AGC kinases RSK, AKT and S6K take place. Examples of crosstalk via AGC kinase redundancy are summarized in Table 1.1.

Table 1.1 Examples of substrates that are phosphorylated by multiple AGC kinases (Mendoza et al., 2011a)

Substrate	Kinase	Substrate	Kinase	Substrate	Kinase
ERalpha	RSK, S6K	BAD	RSK, S6K,AKT	TCP1	RSK, S6K
rpS6	RSK,S6K	YB-1	RSK,AKT	EIF4B	RSK, S6K,AKT
eEF2K	RSK, S6K	GSK3	RSK, S6K,AKT	RANBP3	RSK,AKT
MAD	RSK, S6K	p27 KIP1	RSK, SGK,AKT	AS160	RSK, SGK,AKT

CHAPTER 2

AKT REGULATES EGFR DEGRADATION AND TRAFFICKING BY PHOSPHORYLATING AND ACTIVATING PIKFYVE

A revised version of this work has been submitted for publication. Dr. Ashley Mackey and Dr. Lucia Rameh processed and analyzed the data with phosphoinositides metabolically labeled with tritium. Human mammary epithelial cells immortalized with dominant negative p53, hTERT and over-expressing EGFR was generated by Dr. Michelle Mendoza.

Introduction

EGFR is a major regulator of cell proliferation, growth, survival, metabolism and motility and is over-expressed or inappropriately activated in many cancers (Riese et al., 2007; Yarden and Sliwkowski, 2001). EGFR carries out these functions by activating multiple signaling cascades, including PI3K-AKT, mTORC1-S6K, and ERK-RSK pathways. PI3K, mTORC1 and ERK variably induce distinct cellular functions depending on the cell type, cell-cycle time, and pathway activation level and duration (Mendoza et al., 2011a). In response to EGF stimulation, AKT, ERK and RSK all contribute to the activation of the mTORC1 – S6K pathway. EGFR and these downstream signaling pathways are regulated via a network of feedback and cross-talk mechanisms (Mendoza et al., 2011a).

Receptor endocytosis is a regulatory mechanism that promotes sustained and spatially-regulated signaling by localizing receptors to signaling endosomes and by promoting receptor recycling to the cell surface (Schenck et al., 2008; von Zastrow and Sorkin, 2007; Zoncu et al., 2009). Alternatively, endocytosis can lead to signal attenuation by culminating in receptor degradation. EGFR endocytosis is initiated by EGF binding to EGFR dimers at the plasma membrane (Chung et al., 2010). Stabilization of EGFR dimers promotes EGFR activation and trans-phosphorylation. Active EGFR is ubiquitinated by CBL E3 ligase and recruits the endocytic machinery. Both clathrin-dependent (Goh et al., 2010) and clathrin-independent (Orth and McNiven, 2006; Sigismund et al., 2005) pathways contribute to EGFR endocytosis. Receptor internalization is followed by localization to EEA.1-positive endosomes, where cargos destined for recycling or degradation are separated (Lakadamyali et al., 2006; Leonard et al., 2008). EGFR molecules are recycled back to the plasma membrane from the early endosomes and the limiting membrane of multivesicular bodies (MVBs) in a RAB4- and RAB11-dependent

manner. Recycled EGFRs engage in additional rounds of endocytosis and signaling (Scita and Di Fiore, 2010). Alternatively, PTP1B can de-phosphorylate EGFR at the limiting membrane of MVBs (Eden et al., 2010; Haj et al., 2002). Dephosphorylated EGFRs enter the MVBs lumen via the endosomal sorting complex for transport (ESCRT) complexes (Haglund et al., 2003; Sigismund et al., 2005). These EGFRs are dissociated from signal-transducing molecules and signaling is terminated. These EGFRs are destined for degradation in the lysosomes.

Proteins involved in EGFR sorting and degradation such as EEA.1 and ESCRT proteins are recruited to the endocytic vesicles via their interaction with phosphoinositides. For example, the endomembranes contain phosphatidyl-inositol-3-phosphate (PI3P), which is recognized by Fab1, YOTB, Vac1, EEA.1 (FYVE) domains found in these respective proteins. Fab1 is a phosphoinositide kinase that phosphorylates PI3P to generate PI(3,5)P2. The SAC3 phosphoinositide phosphatase dephosphorylates PI(3,5)P2 at the 5 position to generate PI3P (Ho et al., 2011). In yeast, deletion of Fab1 disrupts cargo sorting to the yeast vacuoles (Odorizzi et al., 1998). The human homolog of Fab1 is called FYVE-containing Phosphoinositide 3-Phosphate (PI3P) 5 Kinase, PIKfyve. The homolog forms a complex with associated regulator of PIKfyve (ArPIKfyve) and SAC3 at the endomembranes. PIKfyve, facilitates and SAC3 inhibits the progression of early endosomes towards MVBs, suggesting PI(3,5)P2 promotes and PI3P inhibits vesicle progression (Sbrissa et al., 2007). However, the regulation of the PIKfyve-ArPIKfyve-SAC3 complex in mammalian cells is not well understood.

Previous work has implicated Type I PI3Ks, which are activated by Insulin and EGF, in the modulation of vesicular trafficking. Mutagenesis of the platelet derived growth factor receptor's (PDGFR's) binding site for the type I PI3K regulatory subunit p85 blocks PDGFR degradation (Joly et al., 1994). PI3K catalytic subunit p110 recruitment to the plasma membrane

accelerates clathrin coat dynamics (Nakatsu et al., 2010). Injection of p110alpha-blocking antibodies causes transferrin to accumulate within the cells, suggesting p110alpha facilitates transferrin recycling (Siddhanta et al., 1998). Wortmannin, a fungal product that inhibits all PI3Ks, also suppresses transferrin recycling *in vivo* and reduces the rate of endosomal sorting in cell free systems (Barysch et al., 2009; Spiro et al., 1996). Two recent developments prompted us to investigate whether PI3K signals through AKT to modulate trafficking: AKT inhibitors were found to increase protein levels of several RTKs and AKT knockdown reduces transferrin and EGF uptake (Chandarlapaty et al., 2011; Collinet et al., 2010).

We investigated the role of AKT in EGFR trafficking and discovered a novel negative feedback loop in which EGF-mediated activation of AKT promotes EGFR progression through the early endosomes and EGFR degradation by activating the PIKfyve. Multiple AKT inhibitors and AKT knockdown reduce EGFR progression through the early endosomes, the rate of EGFR degradation and PIKfyve activity *in vitro* and in cells. Similarly, knocking down PIKfyve or its activator ArPIKfyve or treatment with the PIKfyve inhibitor reduces the rate of EGFR degradation. The reduced rate of EGFR degradation produced by AKT inhibition is rescued by knockdown of the SAC3 phosphatase. Further, expressing the PIKfyve mutant that cannot be phosphorylated by AKT reduces EGFR degradation. We propose a model in which AKT phosphorylates and activates PIKfyve to facilitate EGFR endosomal progression, thereby increasing EGFR degradation and dampening EGFR signaling.

Results

AKT regulates EGFR degradation.

EGF stimulation causes EGFR degradation via delivery to the lysosomes (Scita and Di Fiore, 2010). In order to address the role of AKT in EGFR degradation, we chose to assess EGFR degradation in human mammary epithelial cells (HMECs), which express 4.5×10^5 EGFR molecules per cell (Spangler et al., 2010). This concentration of EGFR is within the range of other cell lines commonly-used in endocytosis studies. HeLa cells express 1.7×10^5 EGFRs per cell (Spangler et al., 2010). MDA-MB-468 and A431 cells, which harbor amplified EGFR, express three and six-fold more EGFR than HMECs, respectively (Filmus et al., 1985; Merlino et al., 1984; Ullrich et al., 1984). Stable over-expression of EGFR in HMECs leads to 25-50% increase in the total EGFR level (Figure 2.1, Panel A), so the approximate number of EGFR molecules is 6×10^5. We used the latter cells for our studies as they were more tractable to interrogation and exhibited a more intermediate EGFR expression level compared to the above mentioned commonly-used cell lines. Stimulation of these HMECs with high concentrations of EGF (100ng/mL) induced a decrease in total cell lysate EGFR levels and this is abrogated by pre-treatment with the lysosomal inhibitor chloroquine (Figure 2.1 Panel B and C). Similar to HeLa cells, 1ng/mL EGF does not cause EGFR degradation, suggesting a specific EGFR activation level is needed to either induce degradation or detect the degradation.

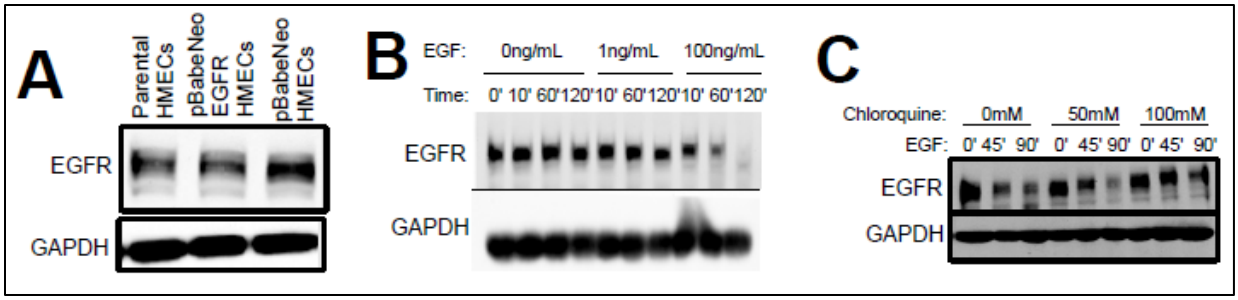

Figure 2.1 EGF stimulation causes EGFR degradation through lysosomes. **A.** Human mammary epithelial cells were infected with EGFR encoding cDNA to moderately over-express EGFR. **B.** HMECs were stimulated with 1 or 100ng/mL EGF. Only 100ng/mL EGF stimulation causes EGFR degradation. **C.** Lysosomal inhibitor chloroquine reduces EGFR degradation in a dose dependent manner.

We hypothesized that any perturbation of EGFR trafficking to the lysosomes would alter its degradation rate. We treated cells with vehicle (DMSO), the dual PI3K-mTOR inhibitor PI103 (Knight et al., 2006) or the highly specific allosteric AKT inhibitor AKTVIII (Lindsley et al., 2005). PI103 interferes with PI3K activity and thus reduces PI(3,4,5)P3, and AKT pS473 and pT308 levels, leading to AKT inactivation. AKTVIII stabilizes an AKT conformation in which the PH and kinase domains are locked together (Calleja et al., 2009). This conformation is proposed to interfere with AKT membrane recruitment and PDK1 and mTORC2 access to AKT. The inhibitor therefore reduces AKT pS473 and pT308 levels and AKT activation. Membrane recruitment of the PH domain containing protein GRP1 is unaffected by AKTVIII suggesting the drug specifically interferes with the AKT membrane recruitment (Green et al., 2008).

In HMECs pretreated with DMSO, EGF stimulation caused more than 80% of the cell's EGFR to be degraded within 120 minutes (Figure 2.2, Panel A). Pre-treatment with AKTVIII and PI103 for 30 minutes sufficiently blocked AKT S473 phosphorylation as expected. Furthermore, pretreatment of cells with these inhibitors reduced the rate of EGF-induced EGFR degradation. A substantial amount of EGFR was detected even after 120 minutes of EGF stimulation. This phenotype was specific to AKT inhibition, as blocking other signaling pathways downstream of EGFR, such as the mTORC1-S6K (Rapamycin) and ERK-RSK (U0126) pathways did not affect EGFR degradation (Figure 2.2, Panel A).

Our observation that both PI103 and AKTVIII stabilize EGFR levels suggests PI3K is working through AKT to regulate EGFR degradation by modulating the trafficking of EGFR-containing vesicles or by modulating EGFR protein synthesis. To test this, we repeated the EGFR degradation assays in the presence of protein synthesis inhibitor cycloheximide (CHX).

Co-treatment of cells with CHX and PI103, AKTVIII, or a structurally distinct ATP-competitive AKT catalytic inhibitor A-443654 reduced EGFR degradation rates (Figure 2.2, Panel B). The effect of A-443654 on EGFR degradation was not as potent as the AKTVIII inhibitor, potentially due to its decreased specificity (Okuzumi et al., 2009). Consistent with our previous experiments, Rapamycin did not alter EGFR degradation. These data indicate AKT controls EGFR degradation in a protein synthesis-independent manner. We further validated AKT controls EGFR degradation by siRNA knockdown of AKT1. Two different AKT1 siRNAs significantly increased the percent of EGFR left undegraded after 120 minutes (Figure 2.2, Panel C). AKTVIII inhibitor reduced EGFR degradation in T47D breast cancer cell line, which has very low EGFR expression (Charafe-Jauffret et al., 2006) suggesting AKT regulates EGFR degradation independent of the EGFR expression level (Figure 2.3).

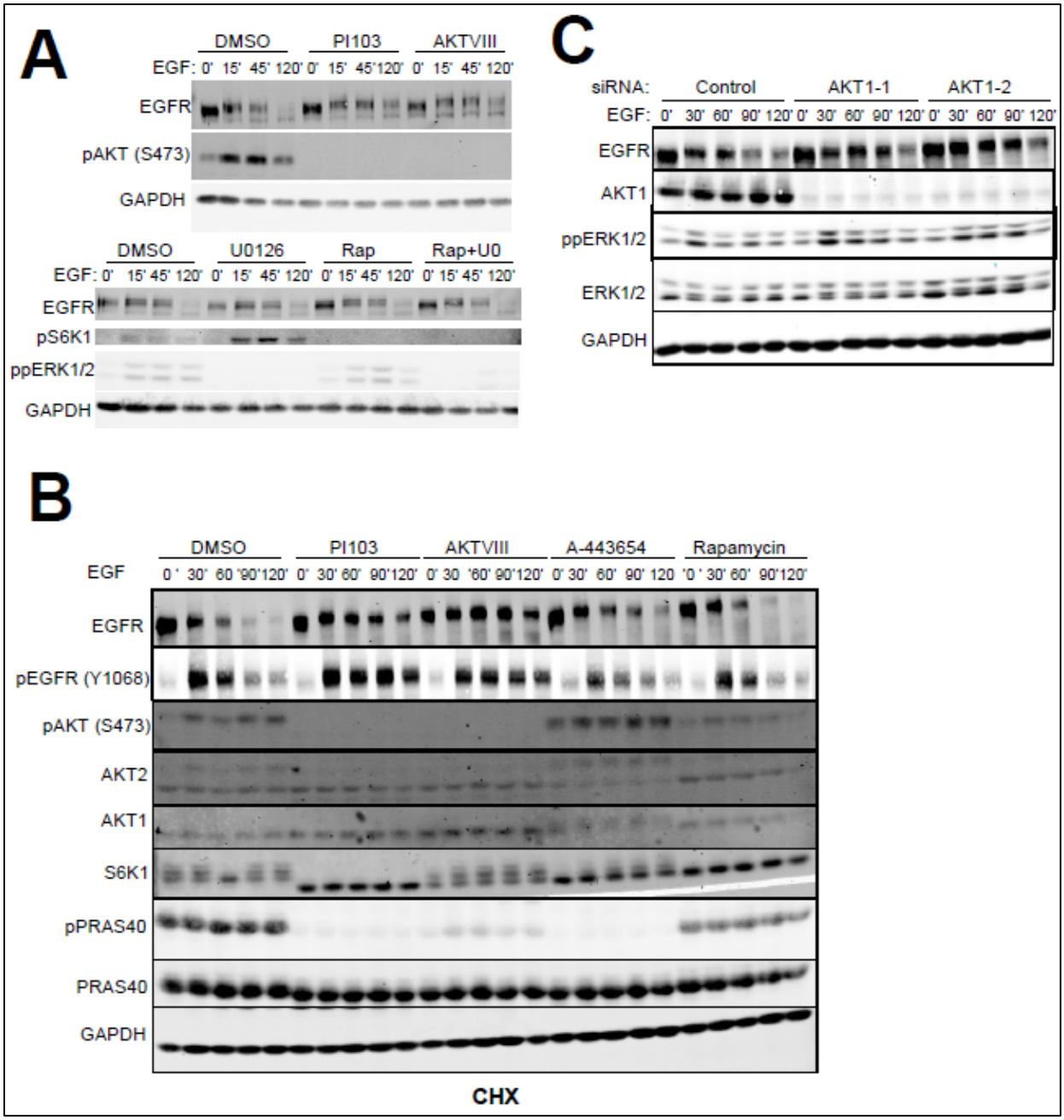

Figure 2.2 AKT facilitates EGFR degradation. **A.** HMECs were starved for growth factors overnight and stimulated with EGF for the indicated amounts of time in the presence or absence of the indicated inhibitors. **B.** Cells were stimulated as above in the presence of 10 μM CHX. Western blot images are representative of at least 3 independent experiments. **C.** Cells were transfected with siRNAs targeting AKT1 or with Non-targeting AllStars control siRNA for 72 hours and deprived of growth factors within the last 24 hours of transfection, treated with CHX for 30 minutes and stimulated with EGF.

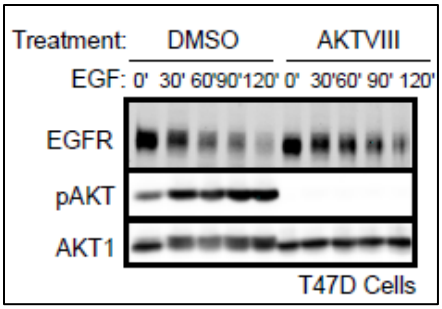

Figure 2.3 AKT facilitates EGFR degradation in T47D cells. Cells were deprived of serum and growth factors and stimulated with 10ng/mL EGF in the presence or absence of AKT inhibitor.

AKT inhibition prolongs EGF-EGFR occupancy in the early endosomes.

Stimulation of cells with PKC or G-protein coupled receptor agonists sequesters EGFR in a peri-nuclear region (Idkowiak-Baldys et al., 2009). This blocks EGF access to EGFR and inhibits ligand-induced degradation. To determine if AKT controls EGFR degradation by facilitating EGF access to EGFR, and the resulting EGFR internalization, we quantified the percent EEA.1 co-localization with Alexa-488 EGF 15 minutes after stimulation using immuno-fluorescence. AKTVIII treatment did not significantly alter EEA.1 co-localization with EGF (Figure 2.4, Panel A). Similarly, AKTVIII did not alter EEA.1 co-localization with EGFR after 30 minutes of EGF stimulation and did not cause EGFR accumulation in the peri-nuclear region (Figure 2.4, Panel B). Together with the observation that AKT inhibition does not prevent EGFR phosphorylation (Figure 2.2, Panel C), these data suggest AKT does not promote EGFR degradation by controlling EGF-EGFR binding or their subsequent internalization.

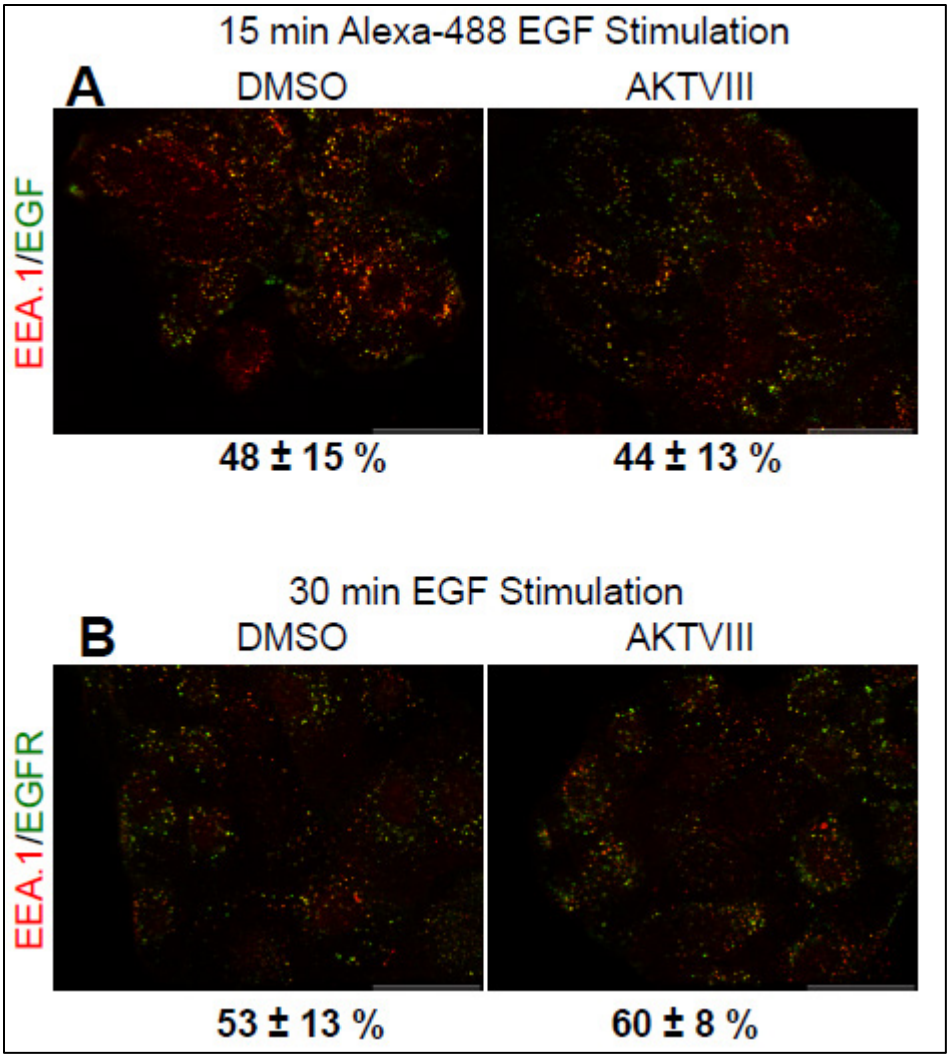

Figure 2.4 AKT inhibitor does not alter EGF and EGFR internalization and localization to EEA.1 positive endosomes. HMECs were deprived of growth factors overnight and stimulated with **A.** 1ug/mL Alexa-488 labeled EGF (green) or **B.** with 100ng/mL EGF and labeled with the early endosomal marker EEA.1 (red) or EGFR (green). Percent co-localization was measured and is not statistically significantly different between two different treatment groups, using bootstrap permutation test (Efron and Tibshirani, 1993).

In order to determine if AKT promotes EGFR degradation by controlling vesicle trafficking, we sought to identify the subcellular location of the EGFR that accumulates upon AKT inhibition at a later time point. In the presence of AKTVIII, EGFR molecules that are not degraded are still phosphorylated (Figure 2.2 C). Because PTP1B dephosphorylates EGFR before EGFR sorts into the lumen of the MVBs (Eden et al., 2010), we hypothesized the un-degraded EGFR accumulates at the early endosomes or the limiting membrane of MVBs. Indeed, AKTVIII treatment increased EEA.1 co-localization with EGF and EGFR 60 minutes after EGF stimulation (Figure 2.5, Panels A and B). To verify the increased EEA.1-EGF and EEA.1-EGFR co-localization indicates increased EGF and EGFR within EEA.1 positive endosomes, we quantified the integrated intensities of the EGF and EGFR signal in each endosome throughout confocal Z-stacks of DMSO and AKTVIII treated cells. We plotted the cumulative probability distribution of endosomes against integrated EGF-EGFR intensity values. In AKTVIII cells, endosomal EGF and EGFR intensities were significantly higher for the same probability value, suggesting AKTVIII treatment increased the amount of EGF and EGFR in each endosome (Figure 2.5, Panels C and D). To synchronize internalization, we stimulated cells with EGF at 4^{o}C prior to moving the cells to 37^{o}C for 60 min. AKTVIII treatment increased EEA.1 co-localization with EGF and EGFR in this case as well (Figure 2.6). Thus we conclude that upon AKT inhibition, un-degraded EGF and EGFR molecules accumulate in EEA.1-positive early endosomes.

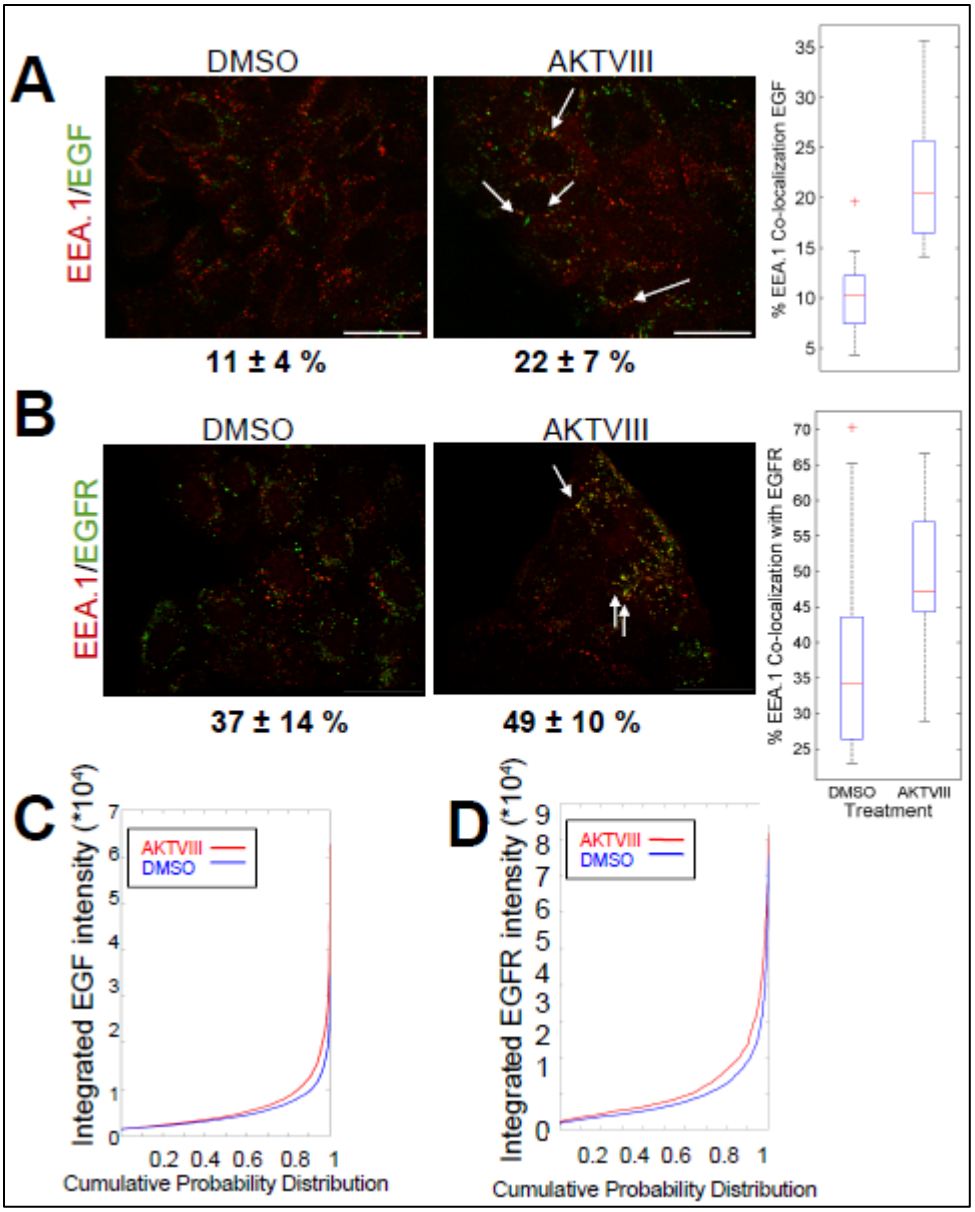

Figure 2.5 AKT inhibition increases EGFR and EGF accumulation at the early endosomes. Cells were stimulated with **A.** 1µg/mL Alexa-488 Labeled EGF (green) or **B.** with 100ng/mL EGF for 60 minutes and labeled with EGFR (green) and EEA.1 (red). EGF/EGFR co-localization with EEA.1 is depicted in yellow and pointed by arrows. Panels to the right are boxplots generated by MATLAB. '+' sign are outliers; whiskers indicate maximum and minimum values. Lower end of the boxes, the middle line and the upper edge of the boxes are 25th, 50th and 75th quantiles, respectively. The median values indicated by redlines are statistically significantly different (p<0.05). **C** and **D.** Cells were treated as in A and B. Cumulative probability distribution of the integrated intensity of EGF and EGFR labeling in each endosome was plotted as detailed in Materials and Methods. AKT reduces EGF and EGFR content of each endosome **C** (n>2300 endosomes .p<10^{-7}) **D** (n>900 endosomes p<10^{-9}). Kolmogorov Smirnov Test was used for calculating statistical significance of the non-normal distributions.

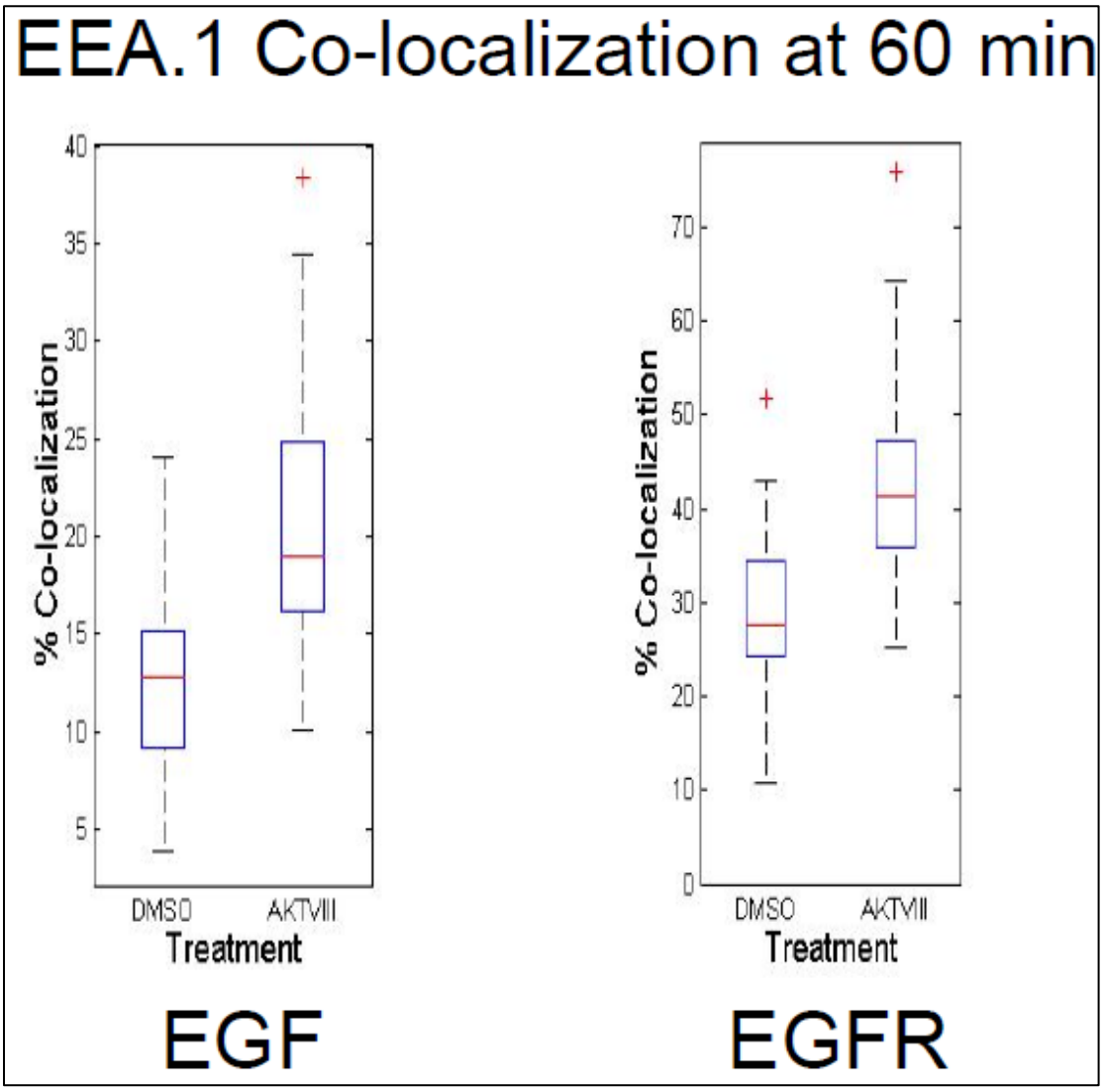

Figure 2.6 AKT inhibitor causes EGF and EGFR accumulation at the early endosomes also when endocytosis is synchronized at 4^{o}C. Cells were stimulated with 1ug/mL Alexa-488 EGF at 4 ^{o}C, washed once with ice cold PBS and translocated to 37 ^{o}C for 60 minutes with pre-warmed media with or without AKT inhibitor. Co-localization of EEA1 (labeled with alexa-568) with EGF (labeled with alexa-488) and EGFR (labeled with alexa-647) was measured, box plots were generated as described in Figure 2.5.

AKT regulates EGFR recycling.

Disruption of receptor recycling by introduction of a dominant-negative RAB4 decreases EGF degradation (McCaffrey et al., 2001). This is thought to be due to the inhibition of continuous rounds of receptor endocytosis in which a subset of the EGFRs would have been degraded with each cycle. We found that Rab11 knockdown reduces EGFR degradation (Figure 2.7). This suggests that interfering with vesicle recycling induces intracellular retention of vesicles, which prohibits additional rounds of EGF binding to EGFR and therefore additional rounds of the recycling and degradation decisions.

We hypothesized AKT facilitates EGFR degradation by promoting receptor recycling and ensuring continuity of the internalization and degradation cycles driven by the EGF in the media. We used an established method to assay receptor recycling in which we measured median cell surface EGFR staining using flow cytometry at different time points: before EGF stimulation (Total), after 15 minutes of EGF stimulation (Pulse) and after washing the EGF-pulsed cells with acid to remove surface EGF and transferring the cells back to 37^{o}C for 10-20 minutes to allow EGFR recycling back to the plasma membrane (Chase) (Sigismund et al., 2008). The percent of recycled EGFR was calculated by the formula 100*((Chase-Pulse)/(Total-Pulse)). We found AKT inhibition reduces the rate of EGF-induced EGFR recycling (Figure 2.8, Panels A and C). Because EGF induces both degradation and the recycling of EGFR, we also assayed EGFR recycling in response to TGFalpha, a distinct EGFR ligand that promotes EGFR recycling without significant degradation (Roepstorff et al., 2009). TGFalpha induced more robust EGFR recycling than EGF and TGFalpha-induced EGFR recycling was also reduced by AKTVIII treatment (Figure 2.8, Panels B and C).

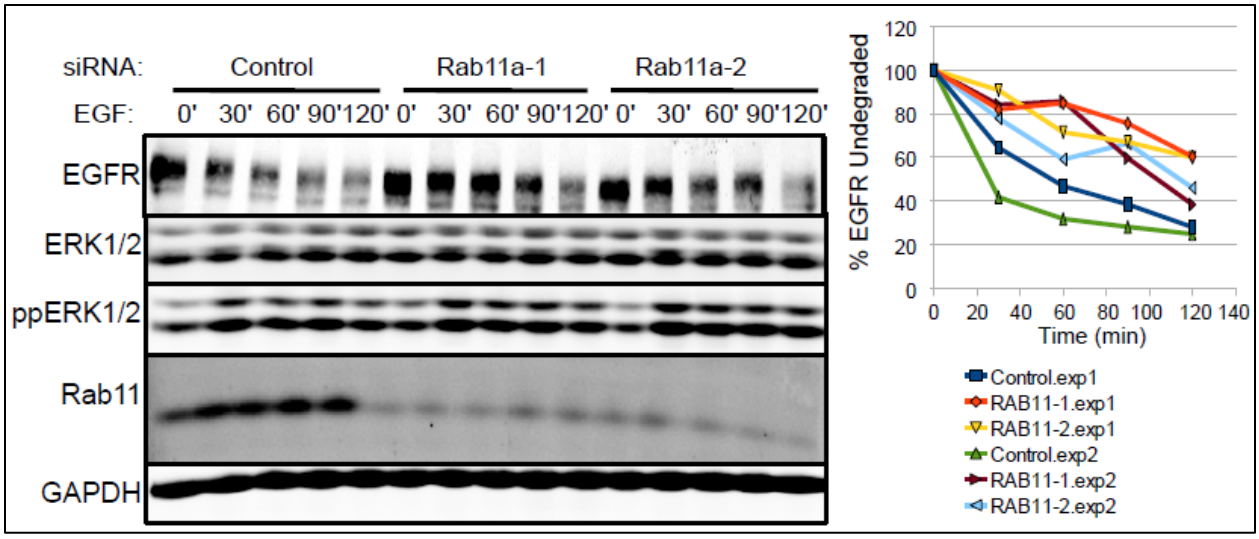

Figure 2.7 RAB11 knockdown reduces EGFR degradation. Cells were transfected with two different siRNAs targeting RAB11. Percent undegraded EGFR was calculated as described in Materials and Methods. Results of two independent experiments are shown in the graph.

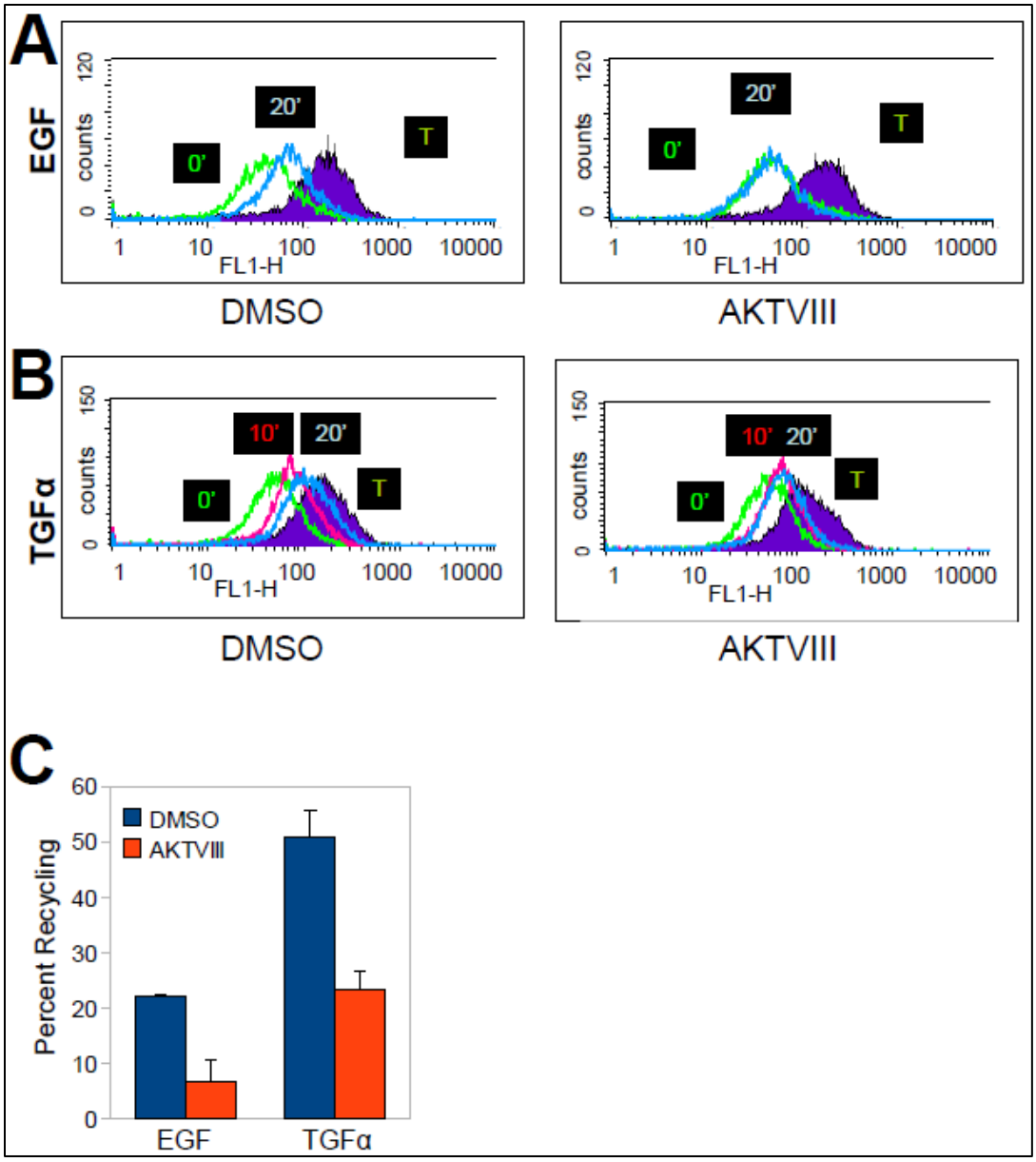

Figure 2.8 AKT facilitates EGF and TGFalpha induced EGFR recycling. Cells were pre-treated with AKTVIII or DMSO for 20 minutes and stimulated with EGF **A.** or TGFα **B.** for 15 minutes. Percent recycling in **C.** was calculated as described in Materials and Methods. Flow-cytometry histograms are representative of 3 independent experiments. Solid purple histograms and T represent total EGFR surface labeling prior to any growth factor stimulation. Green histograms with 0' represent surface EGFR labeling after 15minutesof EGF (100ng/mL) stimulation. Pink (10') and Blue (20') histograms represent surface labeling of EGFR after allowing cells to recycle EGFR at 37^{o}C for 10 and 20 minutes following a pulse of 15 minutes with EGF. **C.** Bar graph represents averages of 3 independent experiments with standard error of mean as error bars. AKT inhibitor causes statistically significant decrease in EGFR recycling by Bootstrap permutation test (p = 0.05). FL-H1: green fluorescence intensity.

AKT regulates EGFR's lysosomal progression.

AKT1 promotes the localization of CD89 targeted antigen to the lysosome associated membrane protein 1 (LAMP1)-containing vesicles (Lang and Lang, 2006; Lang et al., 2001). This suggests that AKT is also involved in lysosomal sorting. To determine if the AKTVIII-induced reduction in EGFR degradation could be due to a reduction in lysosomal sorting, we quantified the percent co-localization of LAMP2 with Alexa-488 EGF. EGF stimulation for 30 minutes caused a small amount of LAMP2 to co-localize with Alexa-488 EGF. However, AKT inhibition did not significantly change the percent LAMP2 co-localization with EGF (Figure 2.9).

The low amount of LAMP2 and EGF co-localization suggests EGF might be getting degraded within the lysosomal compartment. To reduce to loss of our EGF signal, we used chloroquine to inhibit lysosomal degradation (Figure 2.1, Panel C). In the presence of chloroquine, AKTVIII significantly decreased the percent LAMP2 co-localization with EGF, suggesting AKT regulates sorting of EGF towards the lysosomes (Fig. 2.10, Panels A and B). Since EGF causes both EGFR recycling and lysosomal sorting, we also repeated the degradation assay with Betacellulin, an EGFR ligand that induces EGFR lysosomal sorting and degradation without detectable recycling (Roepstorff et al., 2009). Consistent with a role for AKT in EGFR lysosomal trafficking, AKT inhibition reduced the rate of Betacellulin-induced EGFR degradation (Figure 2.10, Panel C).

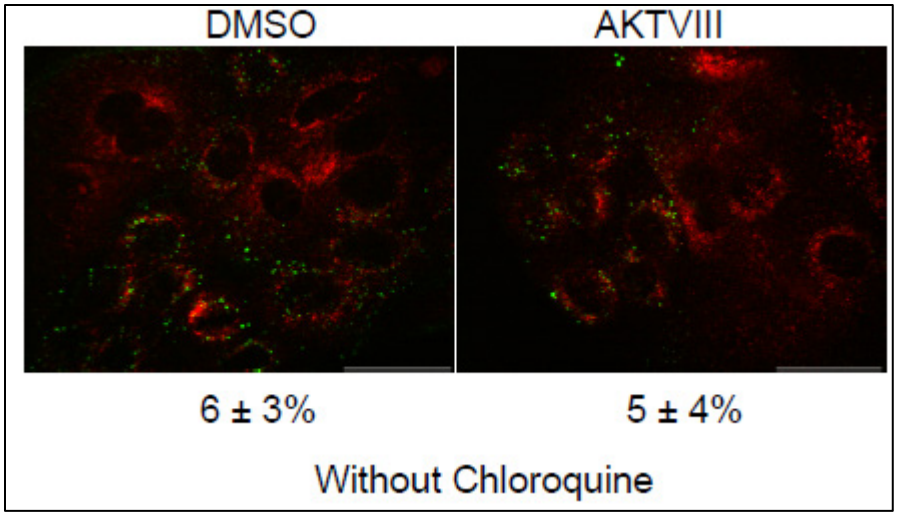

Figure 2.9 AKT inhibition does not affect EGF localization to the lysosomes in the absence of chloroquine possibly due to EGF degradation by lysosomal proteases. Cells were stimulated with Alexa-488 EGF (green) and labeled with the lysosomal marker LAMP2 (red). The average of more than 30 fields of image is given with standard deviations. Average co-localization of LAMP2 with EGF is not statistically significantly different between two treatment groups.

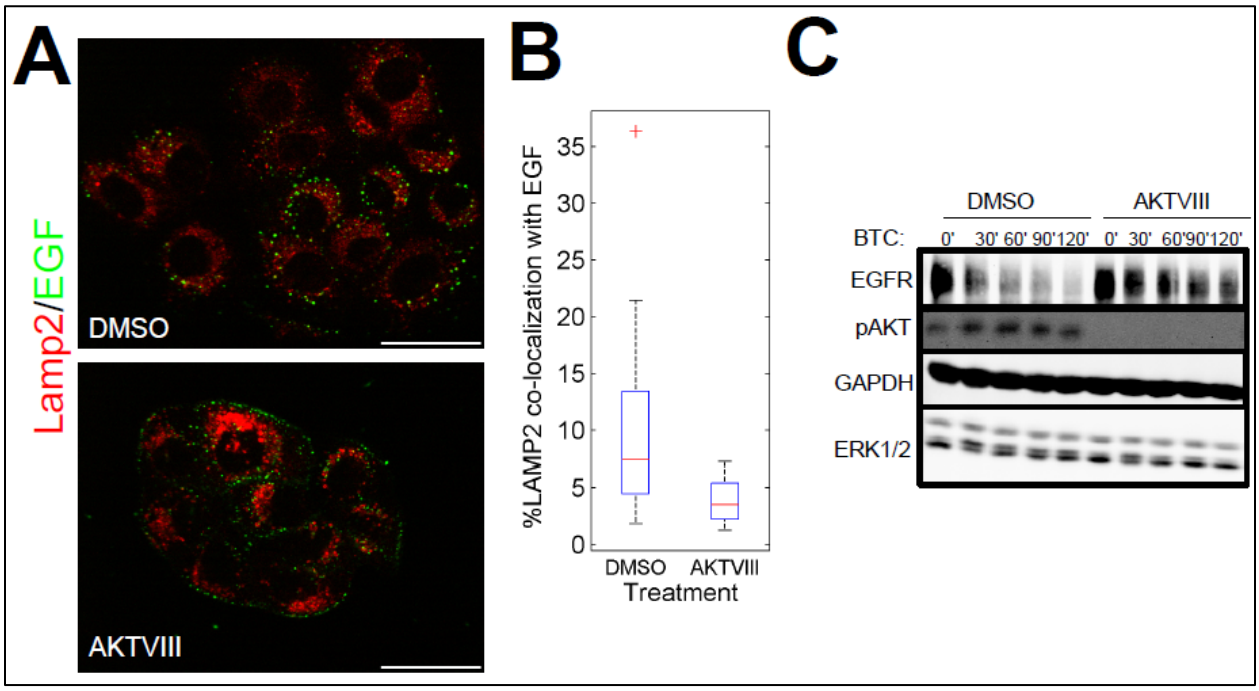

Figure 2.10 AKT regulates EGF localization to lysosomes. **A.** Cells pre-treated with 100mM chloroquine for three hours and stimulated with Alexa-488 EGF (green) and labeled for LAMP2 (red). Yellow represents co-localization. **B.** Quantification and boxplot presentation of LAMP2 co-localization with EGF. AKT inhibitor statistically significantly reduces LAMP2 co-localization with EGF ($p<0.05$) **C.** Cells were starved for growth factors overnight and pre-treated with DMSO or AKTVIII for 45 minutes and CHX for the last 30 minutes of inhibitor treatment and stimulated with Betacellulin for the indicated time points.

Because the observed reductions in total cellular EGFR levels could be influenced by alterations in EGFR ubiquitination (Vivanco et al., 2010), we determined if AKT regulated EGFR ubiquitination. AKTVIII treatment did not alter EGFR ubiquitination (Figure 2.11), consistent with our conclusion that AKT promotes EGFR degradation by regulating EGFR progression from early endosomes towards recycling endosomes and lysosomes.

AKT regulates EGFR degradation by phosphorylating and activating PIKfyve.

We next investigated the mechanism by which AKT controls endocytic vesicle progression. Endosomal identity and ability to progress within the vesicular trafficking system is dictated by the vesicle's phosphoinositide composition (Poccia and Larijani, 2009; Sbrissa et al., 2007). For example, increases in PI(3,5)P2 levels promote the progression of early endosomes into MVBs. Insulin stimulation, which activates AKT, increases PI(3,5)P2 phosphoinositide levels by inhibiting SAC3 phosphatase activity (Ikonomov et al., 2009b). Thus, we tested if AKT functioned upstream of or in a pathway parallel with SAC3 to facilitate early endosome progression and EGFR degradation. SAC3 knockdown rescued the reduction in EGFR degradation induced by AKT inhibition (Figure 2.12, Panel A). When de-convolved, 3 out of 4 siRNAs in the SAC3 siRNA pool reproduced this phenotype, confirming acceleration of EGFR degradation is an on-target effect of the SAC3 siRNAs (Figure 2.12, Panel B). These data indicate SAC3 functions downstream of or in a pathway parallel with AKT to regulate EGFR degradation.

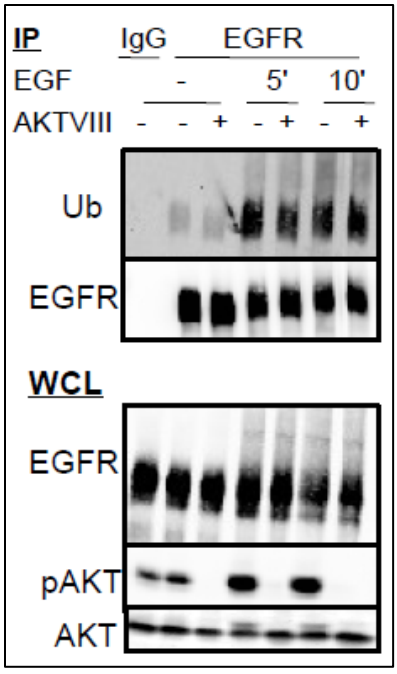

Figure 2.11 AKT does not regulate EGFR ubiquitination. Cells were deprived of growth factors and stimulated with EGF for 5 or 10 minutes. EGFR was immuno-precipitated and labeled with ubiquitin or EGFR antibodies. WCL: whole cell lysate, IP: immuno-precipitation.

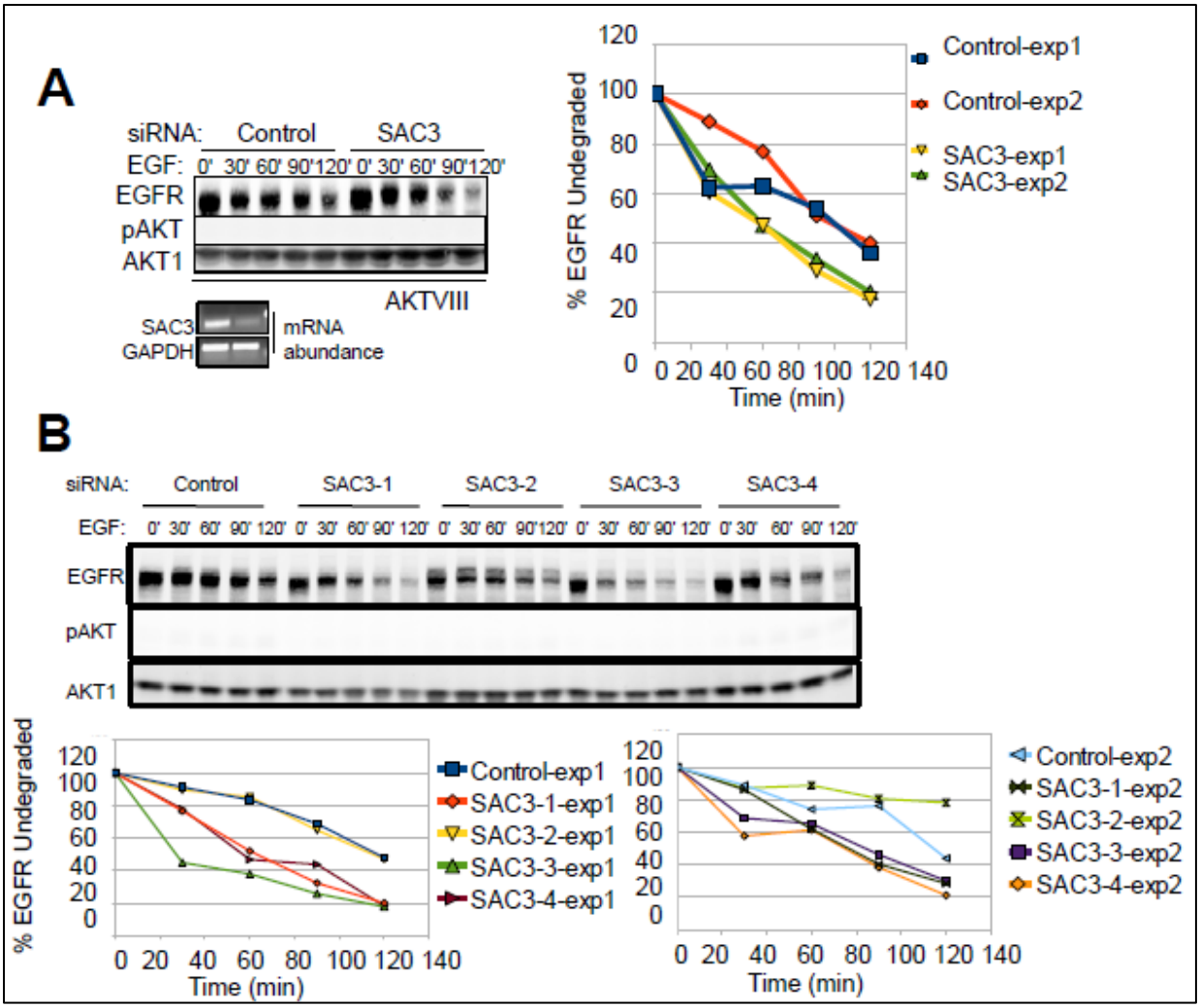

Figure 2. 12 A. SAC3 knockdown rescues AKTVIII mediated reduction in EGFR degradation. Bottom panel: cDNA levels following SAC3 knock down, graph to the left shows quantification for EGFR degradation rates. **B.** siRNA pool de-convoluted: except for SAC3-2 all other siRNAs rescue the reduced EGFR degradation caused by AKTVIII treatment.

Because Insulin stimulation has been shown to inhibit SAC3, we tested if AKT directly regulates SAC3 phosphatase activity (Ikonomov et al., 2009b). We immuno-precipitated the SAC3-ArPIKfyve-PIKfyve complex from human embryonic kidney 293T (HEK 293T) cells pretreated with or without AKT inhibitor. Pretreatment of cells with AKT inhibitor did not change SAC3 phosphatase activity (Figure 2.13). Scansite and PhosphoSite programs do not find a consensus AKT phosphorylation motif on SAC3, collectively suggesting that AKT does not directly regulate SAC3 (Hornbeck et al., 2011; Obenauer et al., 2003).

However, the SAC3-associated PIKfyve was previously found to be phosphorylated by AKT *in vitro* and *in vivo* (Berwick et al., 2004; Hill et al., 2010). To test if PIKfyve regulates EGFR degradation similar to AKT, we perturbed PIKfyve function. Knockdown of PIKfyve or its activator ArPIKfyve, or PIKfyve inhibition using the PIKfyve inhibitor YM201636 (Jefferies et al., 2008) reduced the rate of EGFR degradation (Figure 2.14, Panels A, B and C). Together these results suggest that similar to AKT activity, PIKfyve activity also facilitates EGFR degradation.

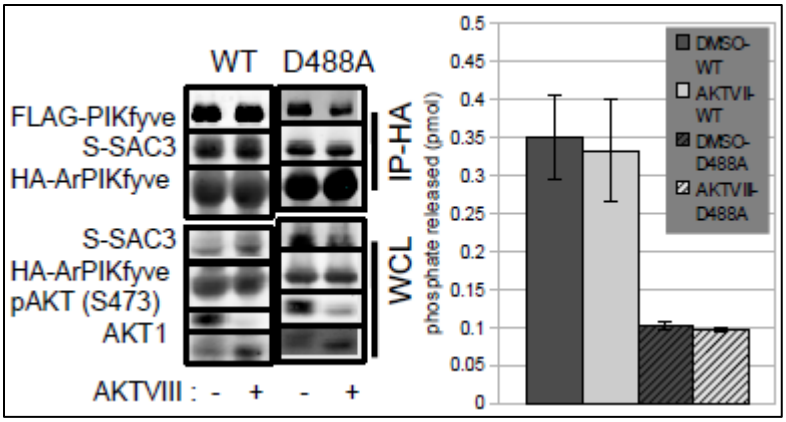

Figure 2.13 AKT does not regulate SAC3 phosphoinositide phosphatase activity. SAC3-ArPIKfyve-PIKfyve complex was immunoprecipitated from HEK 293T cells treated with or without AKT inhibitor and phosphoinositide phosphatase activity of the complex was measured as described in Materials and Methods.

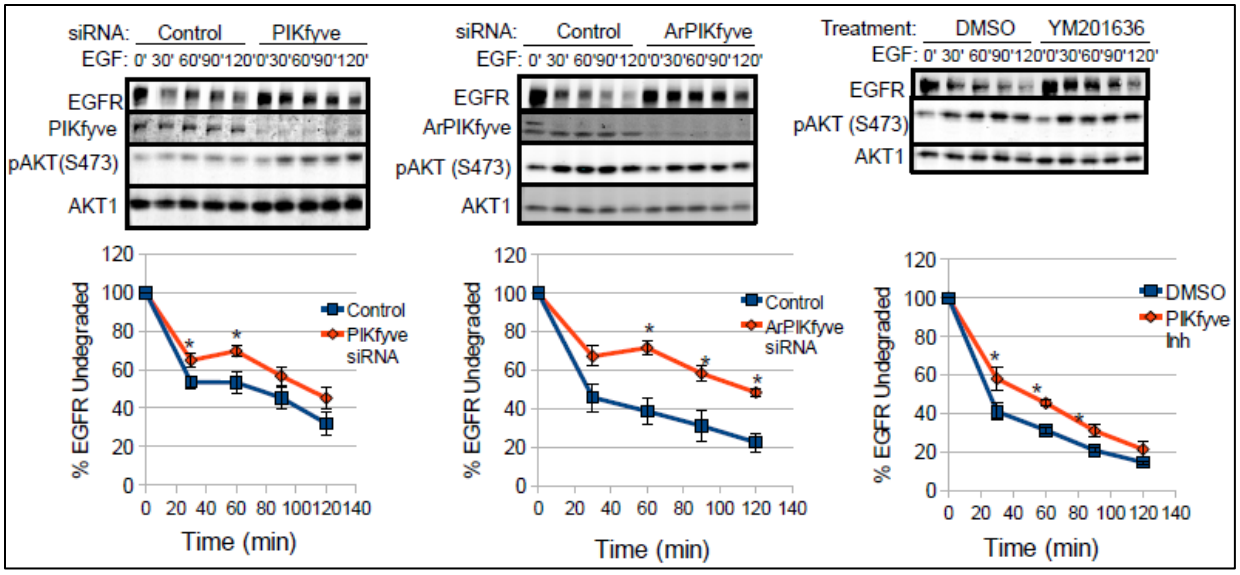

Figure 2.14 PIKfyve regulates EGFR degradation. Cells were transfected with siRNAs targeting PIKfyve, ArPIKfyve or treated with PIKfyve inhibitor YM201636 or DMSO. EGFR degradation was performed and undegraded EGFR was compared to control cells. * indicates statistical significance $p < 0.05$, with Bootstrap permutation test. Error bars are standard error of the mean.

Incubation of AKT with PIKfyve *in vitro* increases PIKfyve activity towards PI3P to generate PI(3,5)P2, suggesting AKT activates PIKfyve (Berwick et al., 2004). However, the effect of mutating the AKT consensus sites on PIKfyve activity is not known. We first confirmed AKT regulates PIKfyve phosphorylation using immuno-precipitated FLAG-PIKfyve transiently expressed in HEK 293T cells. Western blotting with phospho-AKT substrate (pAS) antibody, which recognizes the consensus AKT phosphorylation motifs (Arg-X-Arg-X-X-Ser/Thr) only when the Ser or Thr residues are phosphorylated, detected a band co-migrating with the FLAG PIKfyve protein (Figure 2.15). Both AKTVIII treatment and mutation of one of the AKT consensus phosphorylation site Ser318 to an alanine substantially reduce the pAS signal (Figure 2.15), confirming AKT phosphorylates PIKfyve on S318.

When activated, PIKfyve phosphorylates PI(3)P to generate PI(3,5)P2, which is then dephosphorylated by the myotubularin family of phosphatases to generate PI(5)P. PIKfyve depletion in fibroblasts or treatment with PIKfyve inhibitor results in approximately 85% decrease in PI(5)P levels, suggesting most cellular PI(5)P is generated by the sequential action of PIKfyve and myotubularins (Zolov et al., 2012). In order to test whether AKT inhibition and reduced PIKfyve phosphorylation correlates with PIKfyve activity in cells, we measured 3H labeled phosphoinositide species isolated from HMECs using phosphoinositide extraction, deacetylation and high performance liquid chromatography as previously described (Sarkes and Rameh, 2010). Due to its very low abundance in cells we could not detect PI(3,5)P2 (Sarkes and Rameh, 2010). However, the PI(5)P levels were reduced upon PIKfyve inhibitor treatment confirming that PI(5)P levels in cells reflects PIKfyve activity (Figure 2.16). AKT inhibitor treatment also variably reduced PI(5)P levels suggesting that PIKfyve activity may be regulated by AKT *in vivo*, yet further experiments are required to strengthen this hypothesis.

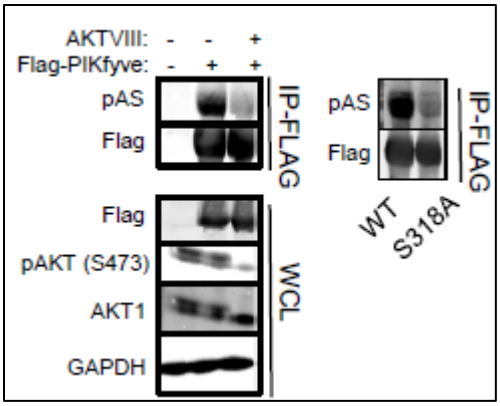

Figure 2.15 AKT activity is required for PIKfyve Ser318 phosphorylation. PIKfyve was immuno-precipitated from HEK293T cells treated with or without AKT inhibitor and phosphorylation of PIKfyve was assessed by western blotting with the phospho-AKT substrate antibody (pAS). Wildtype (WT) but not S318A point mutant is phosphorylated.

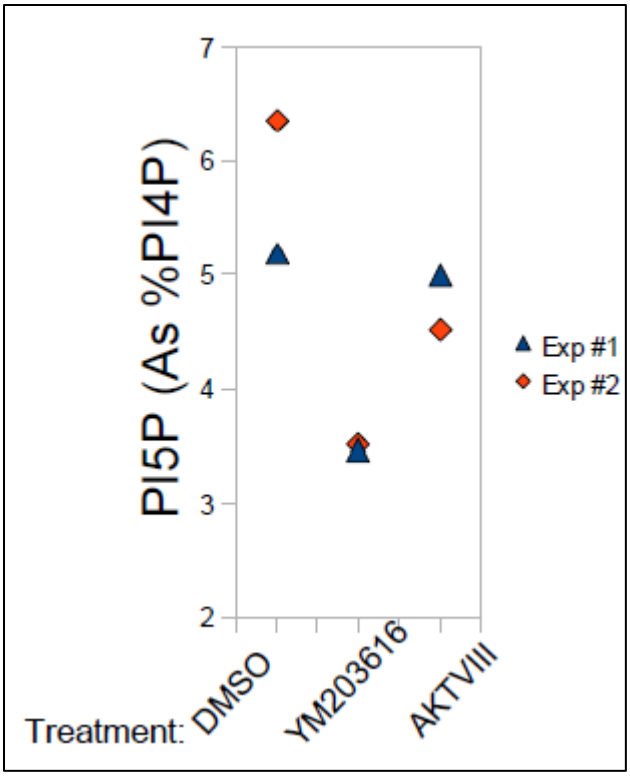

Figure 2.16 AKT and PIKfyve activity regulate PI(5)P levels in cells. HMECs were metabolically labeled with myo-3H-inositols and phosphoinositides were extracted and measured. Depicted is the PI5P abundance from cells treated with different inhibitors as percentage of PI4P abundance. Similar results were obtained when PI5P abundance was compared to total PI abundance. Exp #1 is the first and Exp #2 is the second experiment.

To determine if AKT directly regulates PIKfyve, we tested the effect of AKT inhibition or mutation of the AKT phosphorylation sites on PIKfyve's *in vitro* kinase activity by immuno-precipitating the PIKfyve-ArPIKfyve-SAC3 complex from cells stimulated with or without EGF and pre-treated with DMSO or AKTVIII. The immuno-precipitated complex was incubated *in vitro* with PI3P and ^{32}P-ATP to generate radiolabeled PI(3,5)P2. Resulting radiolabeled phosphoinositides were extracted and separated by thin layer chromatography (TLC) and the radioactive PI(3,5)P2 was quantified by Phospho-Imager. EGF stimulation increased PIKfyve kinase activity which was reduced by pre-treatment of cells with AKT inhibitor, suggesting AKT is required for PIKfyve activation upon EGF stimulation (Figure 2.17, Panel A). Mutation of the Ser318 AKT phosphorylation site on PIKfyve to un-phosphorylatable alanine increased basal PIKfyve activity, but blocked EGF-induced PIKfyve activity (Figure 2.17, Panel B). To test if the S318A mutant's increased basal activity was due to phosphorylation at the second AKT target site, Ser105, we mutated both sites to Alanine. Mutation of both serines mimicked the effect of AKT inhibitor on PIKfyve activity (Figure 2.17, Panel C). Taken together with the observations that AKT regulates PIKfyve phosphorylation and PIKfyve activity in cells, these data suggest AKT directly activates PIKfyve phosphoinositide activity.

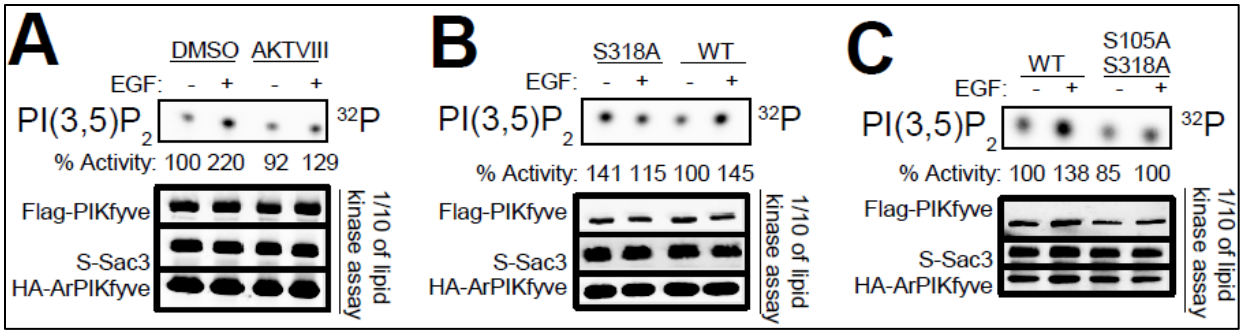

Figure 2.17 AKT phosphorylation activates PIKfyve. PIKfyve complex was isolated from HEK293T cells. 1/11th of the complex was used for western blotting to ensure equal immuno-precipitation of the complex from each condition. The rest of the PIKfyve complex was used in an *in vitro* phosphoinositide kinase assay using PI3P vesicles as a substrate. Resulting radioactively ^{32}P labeled $PI(3,5)P_2$ was seperated by thin layer chromatography and visualized by Phospho-Imager.

To determine the role of AKT phosphorylation of PIKfyve in EGFR degradation, we transiently-expressed wild-type (WT) PIKfyve or the S318A mutant PIKfyve together with SAC3 and ArPIKfyve in HEK 293T cells. Expression of the S318A mutant caused a small but statistically significant reduction in EGFR degradation and increase in phospho-EGFR levels compared to WT PIKfyve (Figure 2.18). Taken together these data suggest that AKT enhances EGF induced EGFR degradation by phosphorylating and activating PIKfyve.

In addition to EGFR degradation, PDGFRβ degradation was also reduced by PIKfyve and AKT inhibitors suggesting that this pathway is likely to be functional in degradation of other RTKs (Figure 2.19).

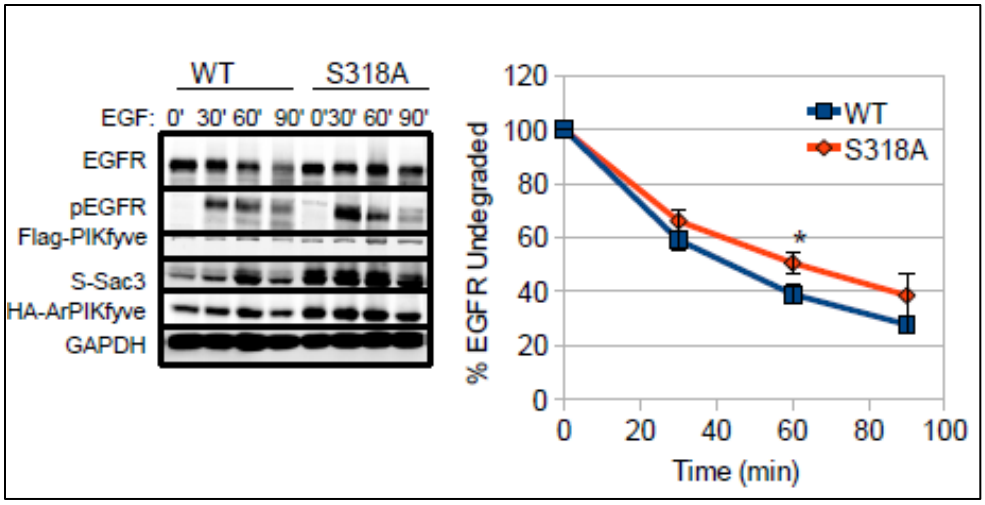

Figure 2.18 Mutation of AKT phosphorylation site reduces EGFR degradation and increases phosphorylated EGFR. HEK293T cells were transfected with WT or S318A mutant PIKfyve and ArPIKfyve and SAC3. EGFR degradation was measured. pEGFR is EGFR phosphorylated at Tyr1068. * indicates statistical significance ($p < 0.05$) measured by bootstrap permutation test. Western blot is representative of at least three experiments.

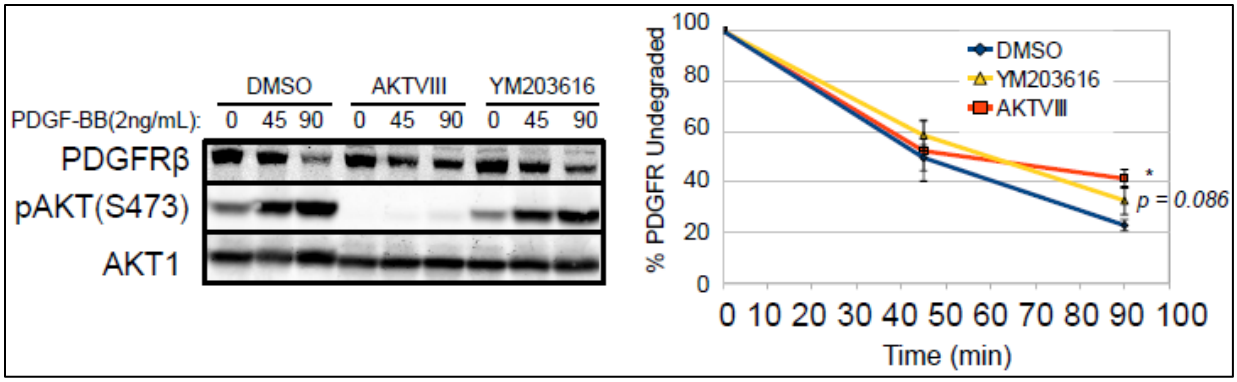

Figure 2.19 PIKfyve and AKT regulate PDGFR degradation. HMECs were deprived of growth factors overnight and stimulated with PDGF-BB in the presence of the indicated inhibitors and PDGFR degradation was quantified. Statistical significance was calculated * indicates statistical significance ($p < 0.05$).

AKT reduces ERK signaling by facilitating EGFR degradation.

Because receptor endocytosis can regulate receptor signaling to downstream pathways, we hypothesized AKT regulated EGFR degradation constitutes a negative feedback loop that reduces EGFR signaling. In this model, EGF stimulation activates EGFR, which activates AKT. AKT directly phosphorylates and activates PIKfyve, which then promotes progression of early endosomes containing EGFR into the degradation path. This activated endocytic trafficking promotes EGFR degradation and would therefore result in reduced EGFR signaling to the RAS-RAF-MEK-ERK-RSK. We tested the functionality of this negative feedback loop in the MCF10A normal breast epithelial cell line, which is a well-established system to study the cross-talk between AKT and ERK pathways (Irie et al., 2005). In these cells, AKT inhibition reduced EGFR degradation and led to more sustained ERK signaling as judged by EGFR, phospho-ERK and phospho-RSK levels following EGF stimulation (Figure 2.20).

To confirm that AKT feeds back to EGFR rather than a parallel pathway that regulates ERK signaling, we tested whether increased ERK activity by AKT inhibition could be suppressed by EGFR inhibition. We treated MCF10As with or without AKTVIII in combination with an EGFR inhibitor. Consistent with the hypothesis that AKT regulates EGFR signaling to modulate ERK activity, EGFR inhibition reduced phospho-ERK levels even in the presence of AKT inhibitor VIII (Figure 2.21).

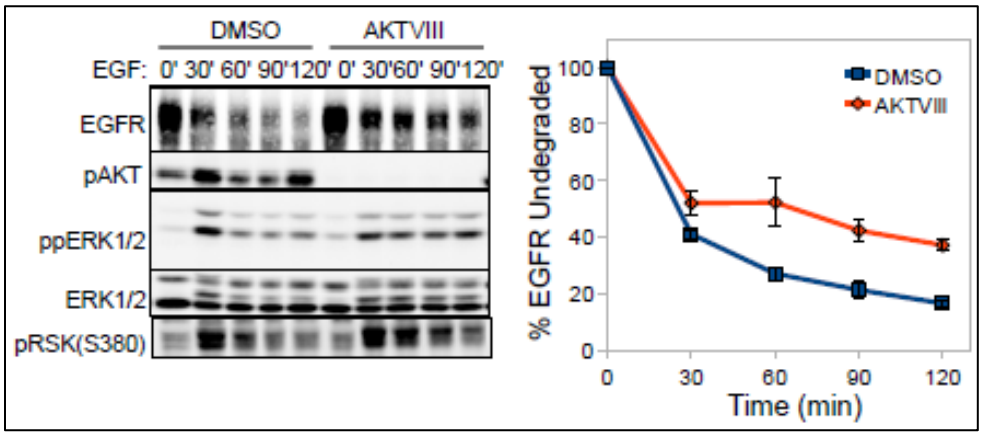

Figure 2.20 AKT facilitates EGFR degradation and reduces ERK signaling. MCF10A cells were stimulated with EGF and EGFR degradation was quantified. The decrease in EGFR degradation upon AKT inhibitor treatment correlates with sustained ERK and RSK phosphorylation.

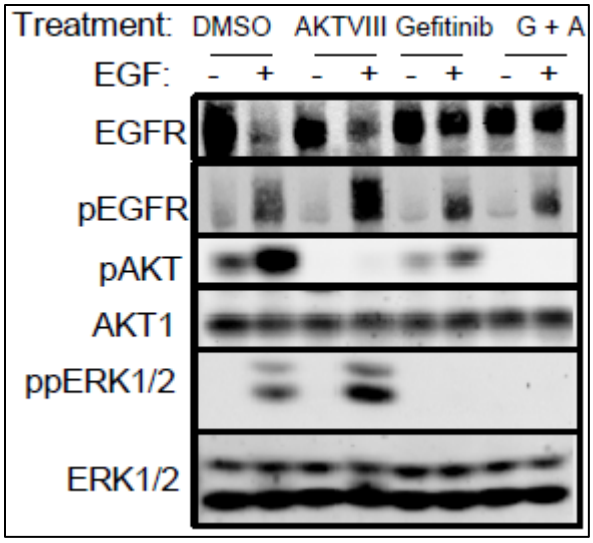

Figure 2.21 ERK activation upon AKT inhibition depends on EGFR activity. MCF10A cells were stimulated with EGF for 120 minutes and ERK phosphorylation was determined in the presence of the indicated inhibitors. Gefitinib: EGFR inhibitor, G+A; Gefitinib and AKT inhibitor co-treatment.

Conclusions and Future Directions

A Role for AKT in EGFR trafficking and turnover

While PI3Ks and their lipid products have been implicated in endocytic trafficking, and trafficking of EGFR, which PI(3,4,5)P3–binding proteins mediate these effects has not been explored. Here we show AKT regulates EGFR trafficking and determined the molecular mechanism behind this regulation (Figure 2.22).

Our results uncover a novel feedback loop by which AKT regulates EGFR degradation. Inhibition of AKT reduces recycling and lysosomal sorting of EGFR, which correlates with an increase in EGFR early endosomal localization and decreased PIKfyve activity *in vitro* and *in vivo*. Reduced AKT activity enhances ERK and RSK activation in an EGFR dependent manner. Thus, we propose a model in which AKT negatively feeds back to EGFR to inhibit EGFR signaling to its downstream pathways: 1) AKT phosphorylates and activates PIKfyve which generates PI(3,5)P2, 2) PI(3,5)P2 facilitates the progression of EGFR-containing early endosomes to MVBs and late endosomes, and 3) EGFR degradation increases and EGFR signaling to ERK decreases. Because AKT is upstream of the general endocytic regulator PIKfyve, this model suggests AKT may regulate the degradation of other RTKs as well. Supporting this hypothesis we found AKT and PIKfyve also regulate PDGFRβ degradation.

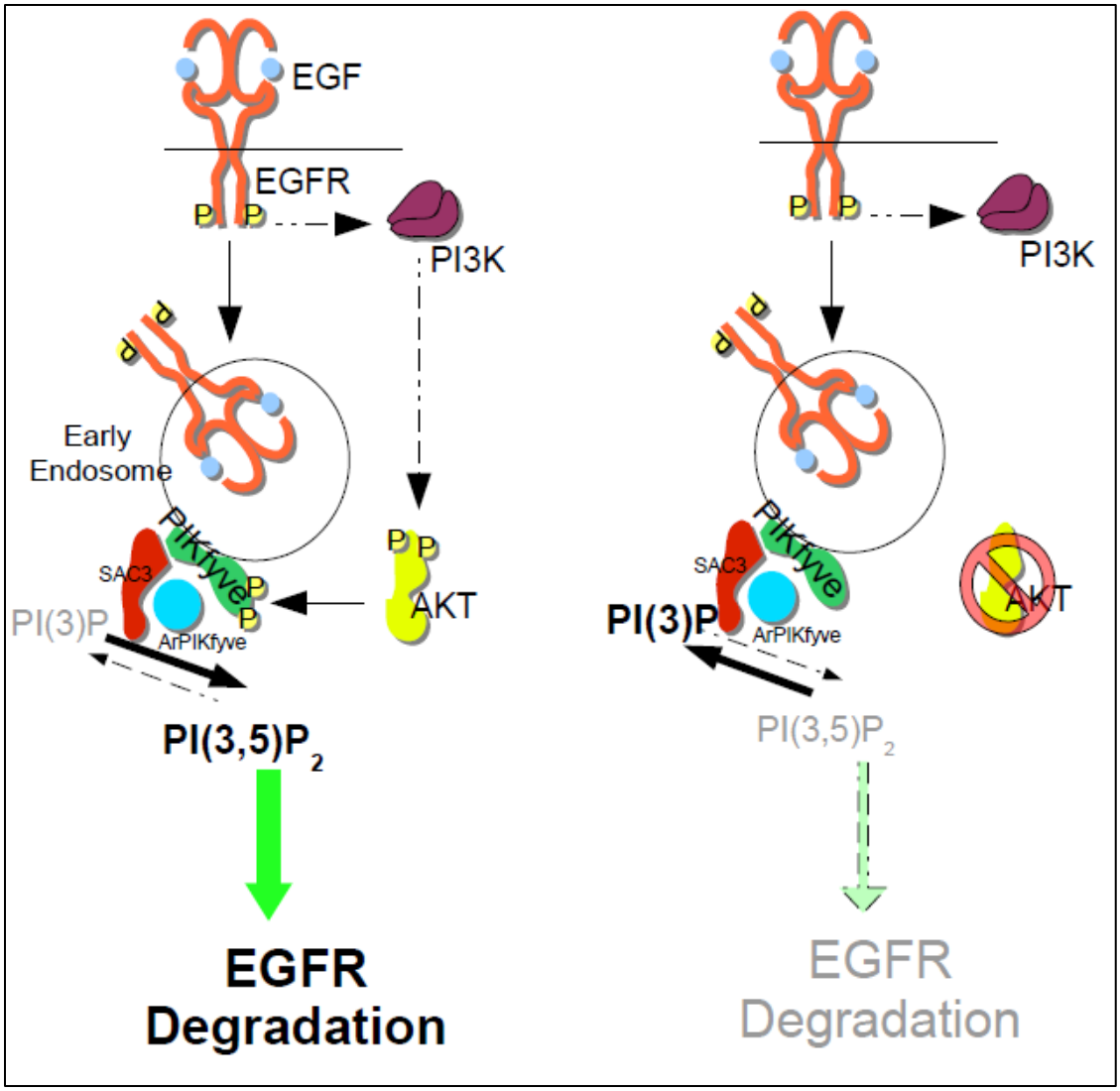

Figure 2.22 Model for AKT mediated EGFR degradation. When AKT is active it phosphorylates and activates PIKfyve to enhance EGFR degradation. When AKT is inhibited EGFR degradation is slowed down due to impaired activation of the PIKfyve complex.

Role for PIKfyve-ArPIKfyve-SAC3 in receptor tyrosine kinase degradation

Previous studies on PIKfyve's role in RTK degradation have been inconclusive. PIKfyve and its product PI(3,5)P_2 regulate retro-grade trafficking and vesicle budding, degradation of Notch and voltage gated calcium channel $Ca_v1.2$ (Ho et al., 2011). Yet, while two structurally-similar PIKfyve inhibitors reduce EGFR degradation and sorting to the lysosomes (de Lartigue et al., 2009; Jefferies et al., 2008), PIKfyve knockdown does not interfere with EGFR degradation in HeLa cells (Rutherford et al., 2006). The different results obtained by different groups could be due to cell type specificity, non-specific effects of the inhibitors or inefficient RNAi knockdown. Alternatively, manipulating expression of one component of PIKfyve-SAC3-ArPIKfyve complex can change the stoichiometry of proteins within this complex and therefore can produce a phenotype different than the one observed by directly inhibiting the kinase activity of an intact complex. For example, in contrast to the aberrant vacuolation observed in PIKfyve inhibition(de Lartigue et al., 2009; Jefferies et al., 2008), expressing a PIKfyve mutant (K2000E) with lowered phosphoinositide kinase activity does not cause a morphological defect unless it is expressed together with SAC3 and ArPIKfyve (Ikonomov et al., 2009a). Similarly, knockdown of SAC3 increases PI(3,5)P_2 (Ikonomov et al., 2009b; Sbrissa et al., 2007), however SAC3 knockout decreases PI(3,5)P2 levels possibly due to disruption of the complex integrity and/or function (Zolov et al., 2012).

Given that relative abundances of these proteins in cells determine the phenotypes observed, we utilized knockdown and exogenous expression together with pharmacological inhibition to determine if PIKfyve-ArPIKfyve-SAC3 complex regulates EGFR degradation. We show SAC3 knockdown, which increases PI(3,5)P_2 levels, rescues the reduction in EGFR

degradation produced by AKT inhibitor treatment. Conversely, knockdown of ArPIKfyve or PIKfyve, both of which are required for $PI(3,5)P_2$ generation, reduces EGFR degradation. Inhibiting PIKfyve kinase activity and expressing the AKT phospho-site PIKfyve mutants which are deficient in phosphorylating PI3P to generate $PI(3,5)P_2$ reduce EGFR degradation. Thus, we conclude PIKfyve regulates EGFR degradation by generating $PI(3,5)P_2$. The observations that PIKfyve inhibitors reduce c-MET (de Lartigue et al., 2009) and PDGFRβ degradation suggest that $PI(3,5)P_2$ and enzymes involved in its metabolism are general regulators of RTK degradation. It is likely that $PI(3,5)P_2$ controls RTK degradation either by recruitment of effector proteins that are involved in endosomal sorting and/or by directly activating ion channels thereby increasing endosome/lysosome fusion events(Dong et al., 2010; Wang et al., 2012)

Regulation of PIKfyve

Despite reducing PIKfyve activity in cells and *in vitro*, AKT inhibitor did not fully inhibit PIKfyve activation in response EGF. PIKfyve can get activated in response to anaplastic lymphoma kinase (ALK) tyrosine kinase. PIKfyve contain many phosphorylated tyrosines, thus whether EGFR can directly activate PIKfyve remains to be determined. It is also possible that EGFR activates another tyrosine kinase, such as Src, to activate PIKfyve. Because both Ser318 and Ser105 follow the AMPK consensus phosphorylation site, it will be interesting to determine whether AMPK can phosphorylate and activate PIKfyve. Another question that remains is how AKT phosphorylation regulates PIKfyve activity. One possibility is AKT phosphorylation regulates PIKfyve association with 14-3-3 proteins as sequence surrounding Ser318 is a moderately strong 14-3-3 binding motif. PIKfyve functions as an oligomeric complex. We did not observe any changes in PIKfyve binding to SAC3 or ArPIKfyve in response to EGF, AKT inhibitor or mutation of the AKT phosphorylation sites suggesting that the stoichiometry of the

trimeric complex is not regulated by AKT. However, whether or not multimeric complex assembly can be regulated by AKT needs to be tested. Another possibility is that AKT regulates PI3P binding by PIKfyve because both of the AKT phosphorylation sites span the FYVE domain of PIKfyve.

AKT substrates involved in receptor recycling

We also found in HMECs, AKT facilitates EGFR recycling. AKT regulates β1 integrin recycling by phosphorylating ACAP-1 and increasing ACAP-1 association with β1 integrin (Li et al., 2005). EGFR and β1 integrin recycling are coupled by RAB coupling proteins (Caswell et al., 2008). However, ACAP-1 over expression does not inhibit EGFR's lysosomal trafficking (Li et al., 2007), suggesting AKT targets involved in receptor recycling are distinct from those involved in degradation. AKT phosphorylates and inhibits AS160 (Kane et al., 2002; Sano et al., 2003), a RAB8 effector that restricts GLUT4 localization to specialized intracellular vesicles. Accordingly, AKT is necessary and sufficient for Glucose Transporter 4 (GLUT4) localization to the cell surface upon insulin stimulation (Ng et al., 2008).

We found that AKT regulates both EGF and TGFalpha-induced EGFR recycling. Current models of EGFR recycling suggest EGF-EGFR complexes are stable at early endosomal pH and their dissociation and subsequent recycling occurs primarily in a slow, RAB11-dependent manner, whereas TGFalpha-EGFR complexes are unstable at the early endosomal pH and their dissociation and subsequent recycling occurs in a fast, RAB4-dependent manner (Sorkin and Goh, 2009). AKT physically associates with RAB11 in 293T cells when stimulated with lysophosphatidic acid (Garcia-Regalado et al., 2008), suggesting AKT may regulate RAB11 or its associated proteins. Thus, in addition to AKT regulating EGFR recycling by facilitating

EGFR exit from EEA.1 positive early endosomes, AKT may directly regulate RAB11-positive recycling endosomes. However, the mechanism by which AKT regulates RAB4 mediated recycling is still not clear.

CHAPTER 3

RSK REDUCES P38-MAPK ACTIVATION AND SIGNALING BY PHOSPHORYLATING AND INHIBITING MEKK3

Work presented in this chapter is not yet published. Analysis of the mass spectrometry experiments was carried out by Dr. Bo Zhai and Dr. Steve Gygi. The analysis of MEKK3 mutations on cell death and proliferation will be carried out by Dr. Michal Nageuic.

Introduction

ERK is a major regulator of cell proliferation, survival and migration. ERK carries out its functions in part by activating the p90 RSK isoforms and MSKs (Cargnello and Roux, 2011). In earlier unbiased studies to identify RSK substrates MEK1-2 inhibitors were utilized (Moritz et al., 2010). Unfortunately MEK1-2 inhibitors can also block ERK5 activation. Additionally, inhibiting ERK1-2 reduces growth factor mediated activation of the RSK related MSK1-2 (Cargnello and Roux, 2011). Given both RSK and MSKs phosphorylate basophilic sites on substrates, discriminating between *de novo* RSK substrates and MSK substrates has been challenging. For example, even though cyclic AMP response element (CREB) phosphorylation in response to mitogens was initially thought to be driven by RSK, studies in MSK1-2 double knock-out mouse embryonic fibroblast demonstrated that MSKs are mainly responsible for this phosphorylation (Arthur and Cohen, 2000). To find *de novo* targets of RSK signaling, we utilized the RSK specific inhibitor BI-D8170 (Sapkota et al., 2007). Using an unbiased mass spectrometry approach, we determined which EGF induced phosphorylation events were sensitive to RSK inhibition. Functional analysis of the changes in the phospho-proteome upon RSK inhibition led us to the discovery of RSK regulation of p38 and ERK5 signaling. We also find that RSK inhibition decreases phosphorylation of MEKK3, a master regulator of both ERK5 and p38. Either RSK inhibition or mutation of the putative RSK target sites increases activation of MEKK3 kinase. We propose that EGF stimulation causes a transient activation of p38 which is terminated by RSK directly phosphorylating and inhibiting MEKK3.

Results

Phosphoproteomic analysis using BI-D1870 identifies RSK specific substrates

To understand the RSK contribution to the phosphoproteome regulated by the EGFR-ERK signaling cascade, we chose a model system whose proliferation relied on EGF. MCF10A cells are non-tumorigenic breast epithelial cells that express EGFR and display robust activation of both ERK and PI3K kinase signaling in response to EGF. We stimulated MCF10A cells with 10ng/mL EGF in the absence or presence of the RSK catalytic inhibitor BI-D1870. Cells were lysed and the lysates were normalized using the Bradford protein assay. Proteins were fragmented using trypsin digestion. Peptides obtained from cell lysates treated with BI-D1870 were labeled with heavy formaldehyde groups whereas the control peptides from DMSO treated cells were labeled with light peptides. Samples were combined, enriched for phospho-peptides using Titanium dioxide and analyzed with liquid chromatography-mass spectrometry (LC-MS/MS). In the final analysis of the mass-spectras we identified 7605 unique phosphorylated peptides.

We then plotted Log2 (BI-D1870/DMSO) ratios for each phospho-peptide. A negative ratio for a phospho-peptide means that the phosphorylation was decreased by BI-D1870 treatment, suggesting that these events required RSK activity. A positive ratio means that BI-D1870 treatment increases the abundance of these phospho-proteins (Figure 3.1). To validate our experimental approach, we first determined the ability of BI-D1870 treatment to reduce the phosphorylation of previously validated RSK targets. Accordingly, SOS1 Ser1134, RAPTOR Ser721, YBX Ser102 and BAD Ser75 (equivalent of mouse Ser112) were all reduced 2 fold or more in response to BI-D1870 treatment. (Table 3.1)

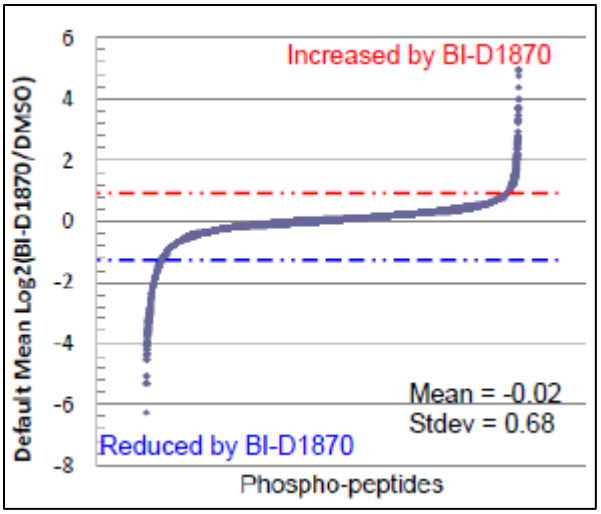

Figure 3.1 RSK regulates EGF induced phospho-proteome. MCF10A cells were stimulated with EGF in the presence or absence of the RSK inhibitor BI-D1870. For each phospho-peptide the change in phosphorylation status due to inhibitor treatment was quantified and plotted as a Log2 ratio. A negative ratio indicates reduction in abundance of a certain phospho-peptide in response to BI-D1870 treatment whereas positive ratio indicates increased phosphorylation in response to BI-D1870 treatment. Dashed lines are Log2 ratios of 1 and -1, which represent two fold increase or decrease in phosphorylation, respectively.

Table 3.1 Analysis of the phospho-proteomic data for known substrates of kinases validates that BI-D1870 specifically inhibits RSK activity. Phosphorylation of validated RSK substrates but not substrates of the related AKT and S6K is decreased by BI-D1870 treatment. BI-D1870 also does not non-specifically reduce phosphorylation of PLK1 substrates.

Protein	Phosphosite	Fold Different	Substrate of
SOS1	Ser1134	Down 4.53 fold	RSK
RAPTOR	Ser721	Down 2.36 fold	RSK
BAD	Ser75	Down 2.38 fold	RSK
YBX-1	Ser102	Down 8.17 fold	RSK, AKT
BAD	Ser99	Up 1.02 fold	AKT, S6K
rpS6	Ser240	Down 1.14 fold	S6K
RICTOR	Ser21	Down 1.16 fold	S6K
PIKFYVE	Ser318	Up 1.1 fold	AKT
PRAS40	Thr246	Down 1.14 fold	AKT
RAC1	Ser71	Down 1.03 fold	AKT
MST2	Ser316	Up 1.1 fold	PLK1
ROCK2	Ser1133	Up 1.12 fold	PLK1

A caveat of using ATP competitive catalytic inhibitors of protein kinases is non-specific inhibition of other kinases due to similarities in their ATP binding pockets. Thus we monitored the effect of BI-D1870 treatment on targets of the related AKT and S6K. We found that despite the reduction in BAD Ser75 phosphorylation, BAD Ser99 (the equivalent of mouse Bad Ser136) phosphorylation, which is targeted mainly by AKT and S6K, was not affected by BI-D1870 treatment. Phosphorylation of rpS6 Ser240-Ser244 and RICTOR Ser21, which are S6K targets, and PIKFYVE Ser 318, PRAS40 Thr246 and RAC1 Ser71 which are AKT targets were also not

altered. Altogether these results suggest that BI-D1870 does not inhibit AKT or S6K. Polo like kinase (PLK) can be inhibited by BI-D1870 *in vitro*, yet at a concentration that is more than 10 fold over the concentration required for RSK inhibition (Sapkota et al., 2007). To determine if BI-D1870 inhibits phosphorylation of PLK substrates in cells, we also monitored the changes in phosphorylation of known PLK substrates. Mammalian sterile 20 like kinase 2 (MST2) Ser316 and Rho associated kinase 2 (ROCK2) Ser 1133 phosphorylation events, both of which have been shown to be mediated by PLK (Lowery et al., 2007; Mardin et al., 2011), remained unaltered by BI-D1870, suggesting BI-D1870 does not interfere with PLK activity under our experimental conditions.

Bioinformatics analysis of RSK phospho-proteome uncovers RSK regulation of p38 and ERK5.

To gain a global understanding of the RSK regulated phospho-proteome, we tested the statistical significance of enrichment for signaling pathways in our data set. We performed the analysis with phospho-proteins whose phosphorylation levels were differentially regulated by RSK inhibition by a factor of 2. Of the 7605 phosphorylation we found that 607 of them were differentially regulated by the drug treatment: Phosphorylation of 427 peptides was down regulated and 180 peptides were up-regulated. Functional clustering of the phospho-proteins using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et al., 2009) into KEGG pathways revealed that RSK regulated proteins were statistically significantly enriched for a variety of biological processes (Figure 3.2). Underlying the importance of RSK in growth factor signaling, phospho-proteins down regulated with BI-D1870 belonged to EGFR and Insulin Signaling. RSK regulated proteins were also enriched for migration pathways such as adherens junction signaling and regulation of actin cytoskeleton regulatory pathways, which is consistent with the idea that the ERK-RSK signaling contributes

to cell migration through not only regulating transcription factors but also regulating cytoplasmic proteins(Mendoza et al., 2011b; Woo et al., 2004).

Surprisingly, RSK inhibition resulted in upregulation of phosphorylation of proteins that clustered into the MAPK pathway. Activation loop phosphorylation of p38 and ERK5 were both increased in response to BI-D1870 treatment (Figure 3.3). We also observed upregulation of Tau phosphorylation on Ser396 and Thr403, which are direct targets of p38 *in vitro* (Reynolds et al., 2000). The activating phosphorylation on MSK2, Ser343 and Ser347, were also increased. Heat shock protein 27kD phosphorylation and TAB3 phosphorylation both of which are known to be regulated by the p38 activated MK2 were also up-regulated (Butt et al., 2001; Mendoza et al., 2008). These data demonstrates that p38 and majority of its downstream signaling components were activated by RSK inhibition.

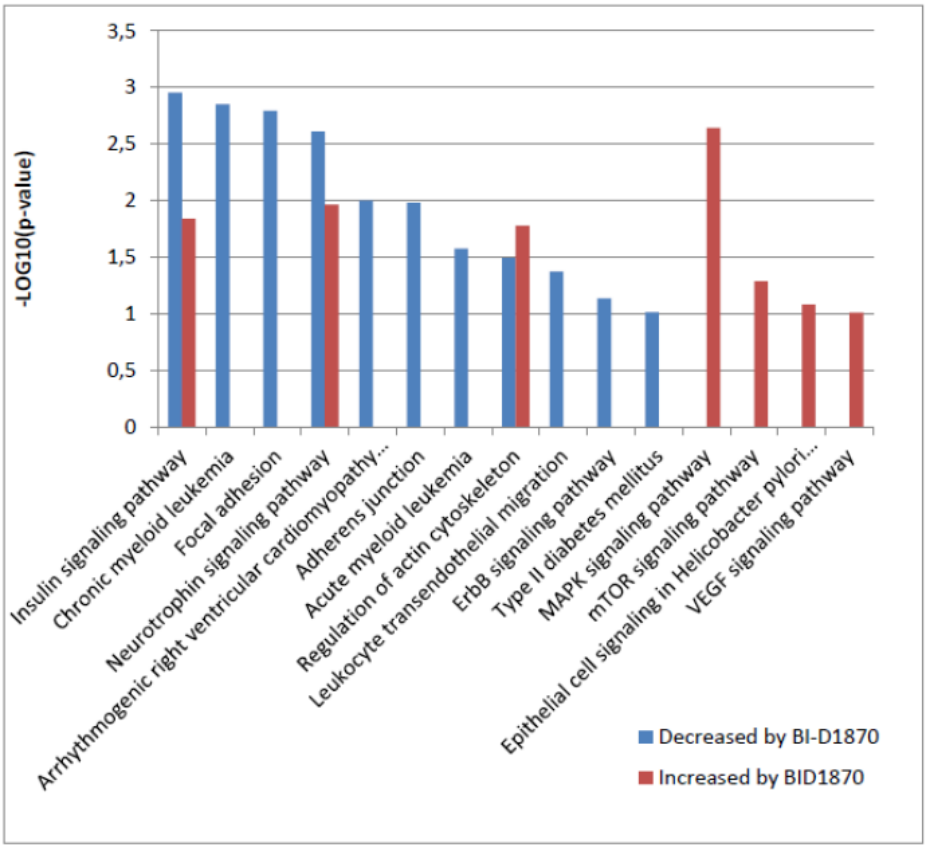

Figure 3.2 RSK regulates multiple signaling pathways. Proteins whose phosphorylation was altered two fold or more in response to BI-D1870 were used for functional clustering using DAVID. Proteins were clustered into KEGG signaling pathways. Signaling pathways that show statistically significant enrichment in the phospho-proteomic data sets with *p*-value of 0.1 or less are depicted above.

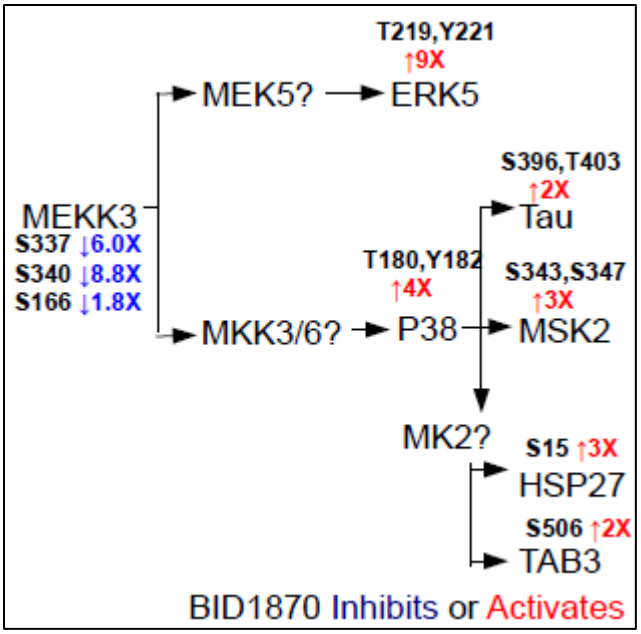

Figure 3.3 BI-D1870 treatment reveals RSK mediated crosstalk to p38 Signaling. Phospho-proteins grouped in the MAPK signaling pathway cluster in the analysis in Figure 3.2 are depicted in this figure. Fold changes in phosphorylation in response to RSK inhibitor treatment is shown with arrows pointing to the direction of change.

In order to validate upregulation of p38 and ERK5 signaling by RSK inhibition, we utilized western blotting. EGF stimulation of MCF10A cells caused a small increase in p38 activation loop phosphorylation which was dramatically increased when cells were pre-treated with BI-D1870. To determine if the increase in p38 activity was due to a potential off target effect of BI-D8170, we used a structurally different RSK inhibitor, SL0-101 (Smith et al., 2005). Similar to BI-D1870, SL0-101 treatment also dramatically increased the p38 phosphorylation in response to EGF. This increase was accompanied by a reduced gel mobility of MSK2 and ERK5 suggesting that these kinases were also hyperphosphorylated. Taken together we conclude that RSK activation downstream of EGF is responsible for reducing p38 and ERK5 signaling pathways (Figure 3.4).

RSK regulates MEKK3 phosphorylation and kinase activity

One possible mechanism of RSK mediated MEKK3 inhibition may be through inhibition of RAC1, the activator of MEKK3. AKT phosphorylates RAC1 on Ser71 and inhibits GTP binding by RAC1, which may lead to reduction in p38 signaling. Because AGC kinases can redundantly phosphorylate certain substrates we asked whether RSK inhibition resulted in decreased RAC1 phosphorylation. Similar to other AKT substrates whose phosphorylation did not change in response to BI-D1870 treatment in our data set, we also found RAC1 Ser71 phosphorylation is not changed (Table 3.1).

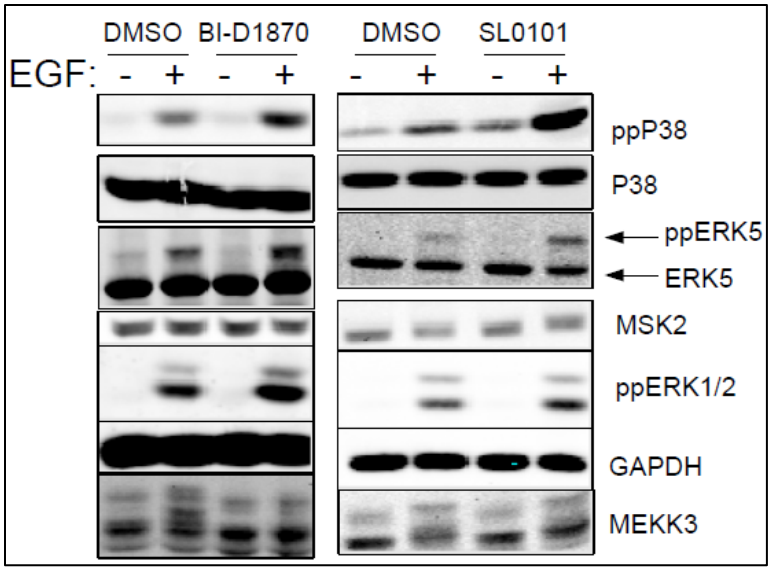

Figure 3.4 RSK inhibition enhances EGF stimulated ERK5 and p38 signaling. MCF10A cells were stimulated with EGF (10ng/mL) in the presence or absence of the indicated inhibitors for 10 min. Treatment with either RSK inhibitor enhances EGF stimulated p38 activation loop phosphorylation as well as ERK5 phosphorylation. Western blots are representative of two independent experiments.

In order to find putative RSK substrates involved in regulating p38 and ERK5 signaling we searched for peptides whose phosphorylation sites resided in the in the canonical RSK target motif Lys/Arg-X-Arg-X-X-Ser/Thr. Out of 7605 phospho-peptides 380 of them contained a putative consensus RSK phosphorylation site. 51 phospho-peptides were down regulated 2 fold or more in response RSK inhibition. Among these proteins MEKK3 Ser337 phosphorylation was decreased 6 fold upon BI-D1870 treatment (Table 3.2).

Table 3.2 Top ten proteins whose basophilic phosphorylation showed highest reduction in response to BI-D1870 treatment

Official Gene Symbol	Site	Fold inhibition
WDR62	Thr50	15.9
MDB4	Ser318	15
AHNAK	Ser315	9.8
DDI2	Ser194	9.5
LRRC41	Ser105	9.5
FAM83H	Ser292	8.8
NCBP1	Ser22	7.4
MAP3K3	Ser337	6.0
LAD1	Ser38	5.8
SIPA1L1	Ser1508	5.7

Phospho-AKT substrate antibody labeling of immuno-precipitated MEKK3 was reduced upon treating cells with BI-D1870 (Figure 3.5). Additionally, EGF stimulation induced a

MEKK3 mobility shift, which was abrogated by BI-D1870 and SL0-101 treatment in MCF10A cells, suggesting that RSK regulates EGF driven MEKK3 phosphorylation (Figure 3.4).

To identify the function of RSK phosphorylation of MEKK3, we aligned the amino-acid sequence of MEKK3 with other MAPKKKs. MEKK3 Ser337 aligned with BRAF Ser429, which is phosphorylated by AKT and PKA (Figure 3.6) (Guan et al., 2000; Konig et al., 2001).

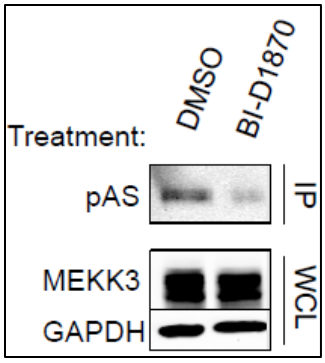

Figure 3.5 BI-D1870 treatment reduces MEKK3 phosphorylation. HEK293E cells were transiently transfected with HA-MEKK3. MEKK3 was immunoprecipitated with anti-HA antibodies from cells treated with or without BI-D1870 and blotted with the phospho-AKT substrate antibody (pAS). Western blots are representative of two independent experiments

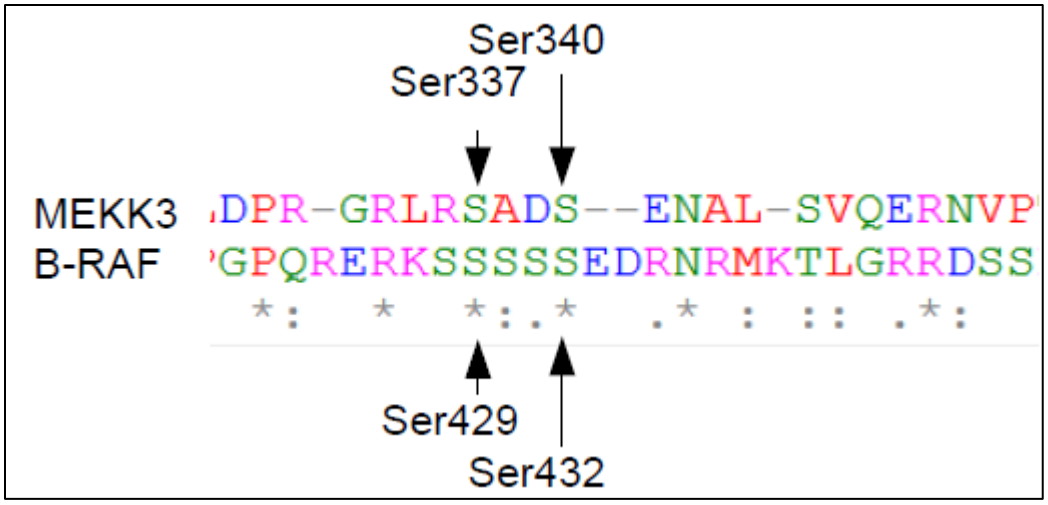

Figure 3.6 BRAF Ser429 aligns with MEKK3 Ser337. Alignment was performed using ClustalW. BRAF phosphorylation of Ser429 is inhibitory for BRAF kinase activity. ":" indicates similarity, "*" indicates identical amino acids.

Phosphorylation of Ser429 reduces BRAF activity *in vitro*, suggesting that MEKK3 Ser337 phosphorylation may also reduce MEKK3 activity. Further analysis of the phospho-proteomic data revealed MEKK3 Ser166 phosphorylation is also down regulated 1.8 fold upon RSK inhibition. To test, if RSK activity inhibited MEKK3, we immunoprecipitated exogenously expressed HA-MEKK3 from HEK293 cells treated with or without BI-D1870 and measured MEKK3 autophosphorylation as well as phosphorylation of one of its downstream targets MKK3. To prevent interference from MKK3 autophosphorylation, we mutated the catalytic Lys in the ATP binding pocket of MKK3 to an Arg (K/R). MEKK3 phosphorylation of MKK3-K/R and MEKK3 autophosphorylation were both enhanced when cells were treated with BI-D1870 suggesting that RSK activity inhibits MEKK3 (Figure 3.7). To determine whether RSK inhibition of MEKK3 was due to MEKK3 phosphorylation of Ser337 and Ser116, we measured the kinase activity of wild-type (WT) and S337A, S166A or the S337 and S166A (AA) mutants of MEKK3. Mutating either or both phosphorylation sites to Ala increased MEKK3 activity suggesting that RSK inhibits MEKK3 activity by regulating the Ser337 and Ser166 phosphorylation (Figure 3.7).

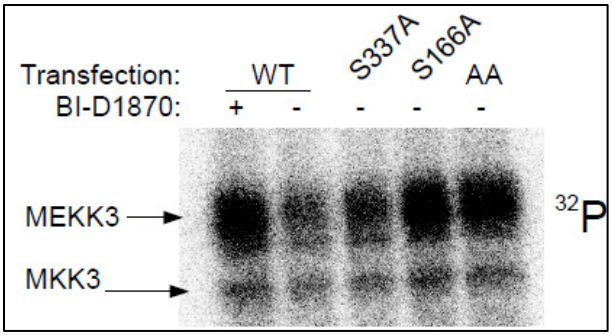

Figure 3.7 RSK inhibition and mutation of the RSK phosphorylation site activates MEKK3 activity *in vitro.* Wildtype (WT) or mutant MEKK3 was immunoprecipitated from HEK293E cells treated with or without BI-D1870. MEKK3 was then incubated with bacterially purified kinase dead GST-MKK3 and radioactive ^{32}P-ATP. MEKK3 kinase activity was assessed by measuring MEKK3 autophosphorylation and GST-MKK3 phosphorylation. Western blots are representative of two independent experiments.

Conclusions and Future Directions

Our phospho-proteomic screen revealed RSK mediated feedback inhibition of p38 and ERK5 pathways through inactivation of MEKK3. Given that MEKK3 phosphorylation sites follow the consensus RSK target motif, it is highly likely that RSK directly regulates phosphorylation of MEKK3. However, without testing RSKs ability to directly phosphorylate MEKK3 *in vitro,* we cannot rule out the possibility of an intermediate kinase to regulate MEKK3 phosphorylation. We also need to identify which RSK isoform is responsible for the MEKK3 phosphorylation as BI-D1870 and SL0-101 inhibit multiple RSK isoforms (Bain et al., 2007).

Mutation of the putative RSK phosphorylation sites increase MEKK3 activity, however the underlying mechanism is not known. Mutation of these sites does not alter 14-3-3 binding to MEKK3 (Matitau and Scheid, 2008) suggesting RSK does not modulate 14-3-3 binding to MEKK3. MEKK3 and MEKK2 activation is known to involve homodimerization (Zhang et al., 2006a). Thus, RSK mediated inhibition of MEKK3 activity may involve regulation of homo or heterodimerization. Alternatively, MEKK3 phosphorylation may differentially regulate its membrane recruitment.

Delineating the mechanism of how EGF activates MEKK3 will also be important as this may give us clues into how RSK can regulate MEKK3. Expression of active form of H-RAS in MCF10A increases GTP loaded RAC correlates with an increase in MKK3, MKK6 and p38 phosphorylation, suggesting that RAS can activate RAC-MKK3/MKK6-p38 signaling axis (Shin et al., 2005a). Extracellular matrix engagement and EGF stimulation of also activates RAC in an EGFR and PI3K dependent manner (Dise et al., 2008; Marcoux and Vuori, 2003; Tamas et al., 2003). Thus, EGF may activate MEKK3 by activating RAC downstream of RAS and PI3K to

activate MKK3/MKK6-p38 signaling pathway. This hypothesis can be tested by determining the ability of cells to activate p38 in response EGF in the presence or absence PI3K and RAC inhibitors or by introduction of dominant negative RAC.

From a physiological perspective, it is also important to understand the function of the RSK regulation of p38 and ERK5 pathways. In our preliminary analysis we observed that MEKK3 null mouse embryonic fibroblasts (MEFs) have a higher proliferation rate than wild type MEFs suggesting that MEKK3 activity may reduce proliferation (data not shown). Additionally, upon depriving the cells of serum, wild type MEFs did not increase in number whereas; MEKK3 null MEFs continued to proliferate. This can either mean that MEKK3 is a negative regulator of cell proliferation or is a positive regulator of cell death. The latter explanation seems more plausible as p38 null MEFs are also resistant to serum deprivation induced apoptosis (Porras et al., 2004). It is possible that under normal growth conditions, RSK phosphorylates and inhibits MEKK3. This can then restrict p38 activation and therefore inappropriate timing of apoptosis (Figure 3.8). When cells are serum deprived, RSK can no longer phosphorylate MEKK3. Hypo-phosphorylated MEKK3 may then activate p38 signaling which would enhance cell death. MEKK3 also activates NFkB signaling which leads to increased cytokine production (Yang et al., 2001). In our phospho-proteomic data set we did not observe any changes in phosphorylation of the NFkB signaling components. However, this may be due to timing of the screen as MEKK3 regulates both immediate transient NFkB activation as well as the late and sustained NFkB activation (Yamazaki et al., 2009). The finding that RSK mediated MEKK3 phosphorylation regulates NFkB signaling would constitute another example of signaling crosstalk. Currently we are working on reconstituting MEKK3 null MEFs with WT MEKK3 or RSK phosphorylation deficient mutant of MEKK3 to test these hypotheses.

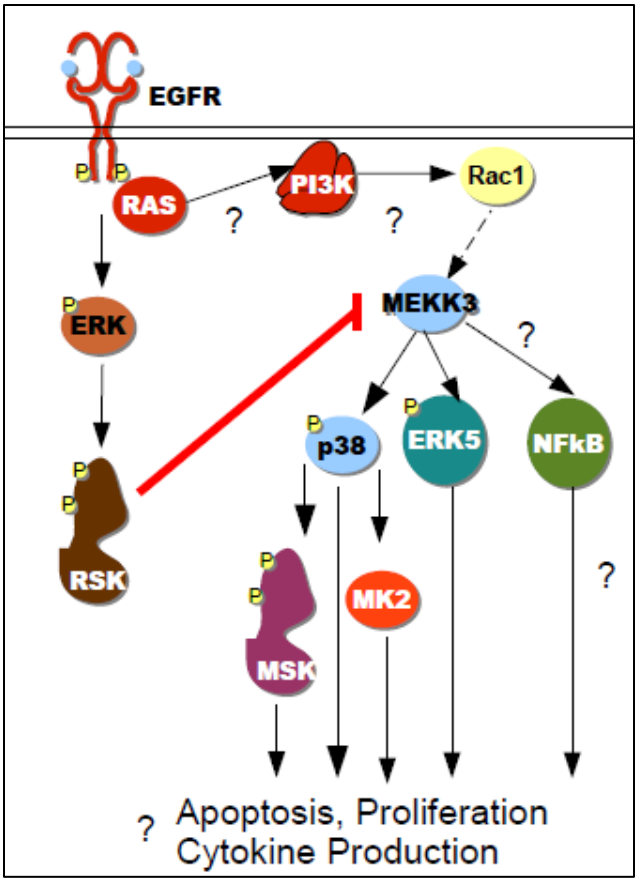

Figure 3. 8 Model of how RSK phosphorylation regulates MEKK3 function and downstream signaling. Upon EGF stimulation RSK phosphorylates and inhibits MEKK3. This prevents p38 and ERK5 activation. However, how EGF activates p38 and ERK5 and how RSK mediated inhibition of MEKK3 regulates downstream biological processes need further investigation.

CHAPTER 4

IDENTIFICATION AND CHARACTERIZATION OF NEW TARGETS OF THE ERK-RSK PATHWAY

Work presented in this chapter is not published. ERK2 D and ERK2 DEF domain expressing MCF10A cells were generated in collaboration with Didem Ilter. Sample preparation and the data analysis for mass spectrometry experiments were carried out by Dr. Bo Zhai and Dr. Steve Gygi. Didem Ilter is carrying out transcriptome analysis to be correlated with the mass spectrometry data. Dr. Michal Nageuic is currently following up on the MELK kinase.

Introduction

ERK is a Ser/Thr Kinase and has been shown to phosphorylate more than 100 proteins in regulation of various biological processes (Yoon and Seger, 2006). The substrate specificity of ERK kinases is achieved by substrate localization, scaffolding, phosphorylation motifs and docking interaction. ERK phosphorylates proteins with the target motif (Pro-X-Ser/Thr-Pro). ERK also utilizes two domains on substrate proteins. Docking site for ERK FXFP (DEF) domains are small amino acid stretches of Phe-X-Phe-Proline. These domains have been identified on ERK substrates involved in migration such as Vinexin (Mitsushima et al., 2004), Paxillin(Ishibe et al., 2003) and WAVE-2 (Mendoza et al., 2011b), and transcription such as FRA-1, ELK-1 and FOS (Dimitri et al., 2005; Murphy et al., 2002; Shin and Blenis, 2010). The other substrate recognition domain is the D domain, which consists of Lys/Arg-Lys/Arg followed by a stretch of 2-6 amino acids then with two hydrophobic residues separated by one amino acid. ERK interacts with DUSP, RSKs and MSKs through D domains on these substrates (Figure 4.1). Importantly, mutation of residues on ERK responsible for interacting with DEF and D domains greatly reduces the ability of ERK to phosphorylate its substrates *in vitro* and in cells. For example, ERK DEF domain binding mutant Tyr-261-Ala (Y261A) cannot phosphorylate FOS but can still phosphorylate RSK, while ERK D domain binding mutant Asp-319-Asn (D319N) cannot phosphorylate RSK but is able to phosphorylate FOS (Dimitri et al., 2005).

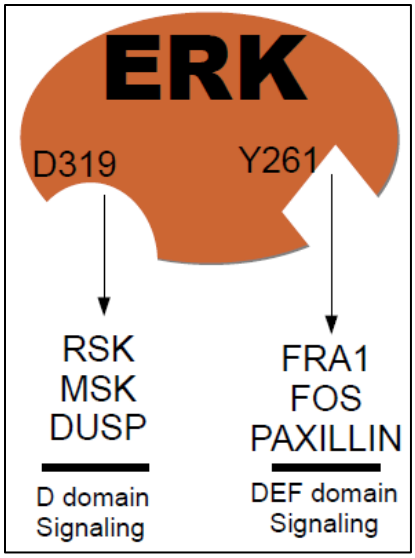

Figure 4.1 Spatially separate docking domains regulate ERK phosphorylation of distinct substrates

Mutation of these spatially separate docking domains on ERK2 in MCF10A cells causes distinct cellular phenotypes. Expression of the ERK2 D319N mutant increases cellular migration and invasion along with enhanced expression of epithelial to mesenchymal transition (EMT) markers and increased stem cell makers. ERK2 D319N induced EMT is FRA1 dependent as silencing of this DEF domain target transcription factor reduces EMT (Shin and Blenis, 2010). On the other hand expression of the ERK2 Y261A mutant, which cannot directly phosphorylate and stabilize FRA1, results in proliferation (Dr. Chris Dimitri unpublished data) but not EMT, invasion and migration. Taken together these data suggest that signaling downstream of spatially separate ERK2 docking domains mediate distinct cellular phenotypes through activation of different group of signaling proteins.

To test this hypothesis we utilized an unbiased mass-spectrometry based approach to study the phospho-proteome downstream of ERK DEF signaling (ERK2 D319N) and ERK2 D signaling (ERK2 Y261A). We had two major goals 1) to identify novel ERK and RSK substrates 2) to understand crosstalk between ERK and other signaling pathways in the context of these mutants. We found many phosphorylation events were differentially regulated by ERK2 DEF and ERK2 D signaling in response to EGF at different time points. Classification of the phosphorylation motifs revealed a decrease in phosphorylation of known RSK substrates in ERK2 DEF cells. We further characterized phosphorylation of two proteins, nuclear interacting partner of ALK (NIPA) and RALBP1 associated EPS domain containing protein 1 (REPS1). Reduced RSK substrate phosphorylation correlated with reduced RSK2 phosphorylation in ERK2 DEF cells but RSK1 phosphorylation was similar in both ERK2 DEF and ERK2 D cells. We also observed that ERK2 D cells had higher mTORC1 and AKT3 pathway activation compared to ERK2 DEF cells. Further analysis of the phospho-proteomic data set and

characterization of these new ERK targets will certainly enhance our understanding of the ERK-RSK signaling pathway and how it interacts with other signaling cascades.

Results

Phospho-proteomic approach identifies ERK2 D and ERK2 DEF signaling specific substrates

To determine the phospho-proteome regulated by ERK2 D and DEF signaling we generated MCF10A cells with endogenous ERK2 knockdown followed by a rescue with ERK2 D signaling (ERK2 Y261A) or ERK2 DEF signaling (ERK2 D319N). These cells were stimulated with or without EGF for 10 or 60 minutes. Due to the complexity of this system, instead of the SILAC approach we used in defining the mTOR phosphoproteome (Yu et al., 2011), we used reductive dimethylation combined LC-MS/MS (Khidekel et al., 2007; Villen and Gygi, 2008). We labeled and combined phospho-peptides obtained from serum starved or EGF stimulated cells. This combination was carried out for both the ERK2 D and ERK2 DEF cells which resulted in 4 distinct EGF regulated phospho-proteomes: 1) 10 min EGF in ERK2 DEF cells, 2) 10 min EGF in ERK2 D cells 3) 60 min EGF in ERK2 DEF cells and 4) 60 min EGF in ERK2 D cells (Figure 4.2). In the 10 minute datasets we identified a total of 12603 phospho-peptides, 5623 of which was identified in both ERK2 DEF and ERK2 D screens. In the 60 minutes datasets we identified a combined 9727 phosphopeptides 4439 of which were identified in both ERK2 DEF and ERK2 screens.

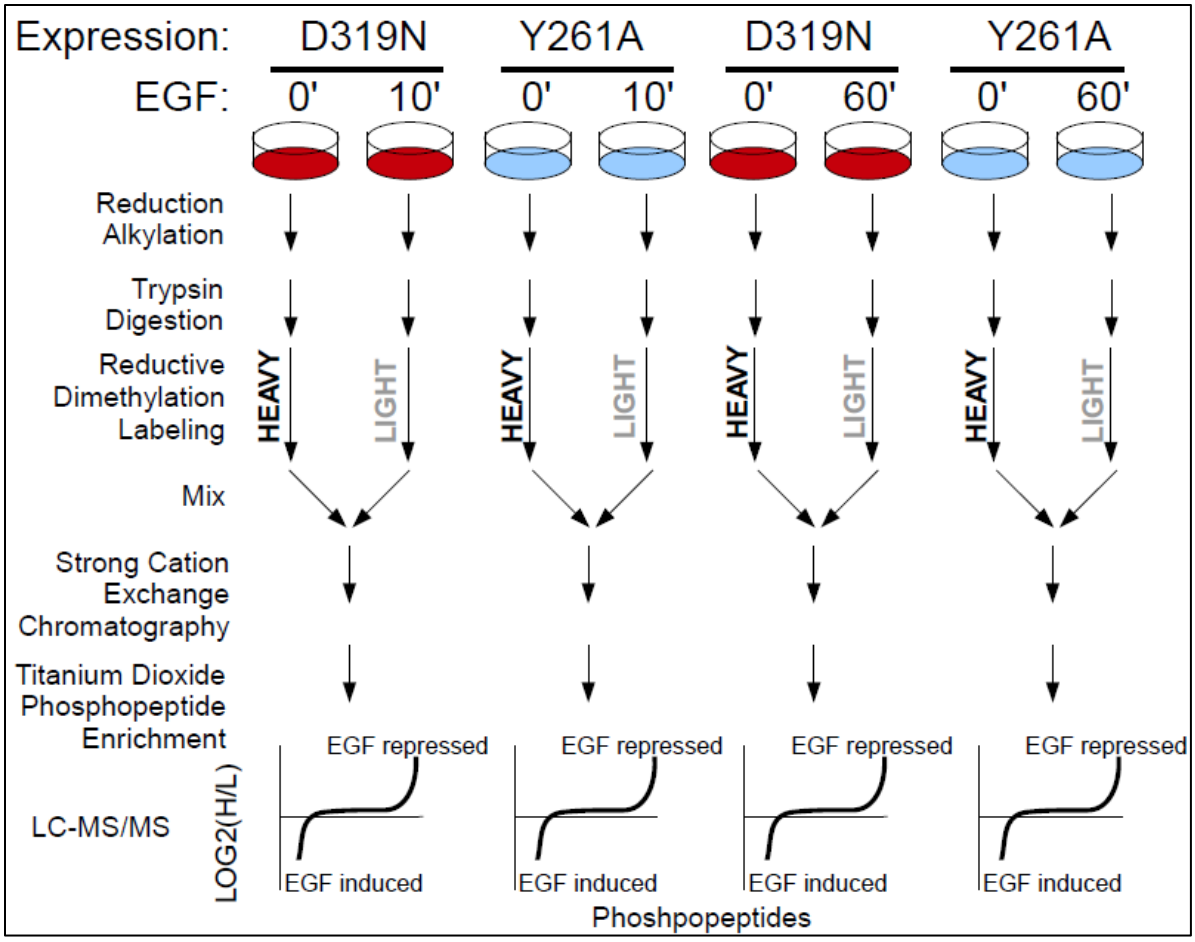

Figure 4.2 Schematic representation of generating phosphoproteomic data in ERK2 D and DEF cells

Because ERK2 DEF cells cannot signal to RSK, we hypothesized that RSK substrate phosphorylation in response to EGF would be reduced in these cells. Accordingly, phosphorylation of several established RSK substrates were increased to greater extend in ERK2 D cells than ERK2 DEF cells, 10 minutes following EGF stimulation. These phosphorylation events included, EIF4B Ser420, GSK3A Ser21 and rpS6 Ser235-Ser236 (Table 4.1). In addition to these known targets, we identified REPS1 Ser709 and NIPA Ser407 phosphorylation to be specifically increased in ERK2 D cells suggesting these may be RSK targets. On the other hand, we could not identify any known effectors of ERK2 DEF signaling in our cells either at 10 minutes or 60 minutes following EGF stimulation in cells. This might be due to the fact that most of the known ERK2 DEF domain targets are low abundance transcription factors.

Table 4.1 ERK2 D but not ERK2 DEF signaling cells promote RSK substrate phosphorylation in response to EGF stimulation. Values in the table below are fold induction in response to EGF stimulation for each time point and each mutant cell line. N/A: Not available.

Mutant	ERK2 DEF	ERK2 D	ERK2 DEF	ERK2 D
Time (min)	10	10	60	60
Substrate				
EIF4B Ser420	1.16	5	N/A	N/A
GSK3A	1.21	2.21	1.05	1.63
RPS6 Ser235	1.04	8.08	0.67	0.87
RPS6 Ser236	1.35	7.69	0.64	1.15
REPS1 Ser709	1.39	2.83	N/A	N/A
NIPA Ser407	1.47	5.05	N/A	N/A

Validation of ERK2 D signaling targets

We determined to validate two of the phosphorylation sites we found in our screens. REPS1 is a highly phosphorylated protein that associates with Ral GTP binding protein (RALBP1) and intersectin (Dergai et al., 2010; Yamaguchi et al., 1997). To determine if our

phospho-proteomic approach identified the correct phosphorylation site on REPS1, we expressed either WT or S709A mutant of FLAG tagged REPS1 in HEK 293 cells. When immune-precipitated from cells western blot analysis revealed that only WT but not S709A REPS1 was labeled with pAS antibody suggesting REPS1 is phosphorylated on Ser709 in cells (Figure 4.3).

To determine if this phosphorylation depends on ERK and downstream RSK activity, we transfected HEK 293 cells with WT REPS1, serum starved cells and stimulated with Phorbol 12-myristate 13-acetate (PMA). REPS1 phosphorylation was detected only in cells stimulated with PMA and not in starved cells. Furthermore, REPS1 phosphorylation was sensitive to MEK inhibitor U0126 and RSK inhibitor BI-D1870, suggesting ERK and RSK mediate Ser709 phosphorylation (Figure 4.4).

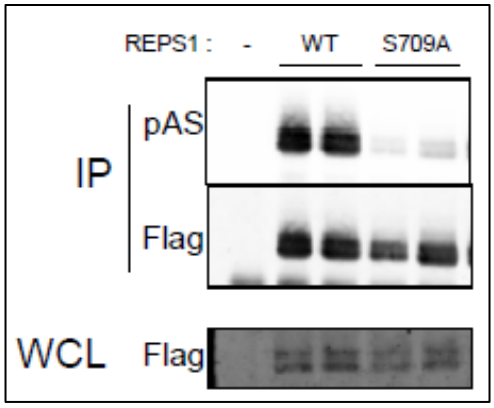

Figure 4.3 REPS1 is phosphorylated at Ser709 in cells. HEK293E cells were transfected with Wildtype (WT) or S709A mutant of FLAG tagged REPS1. REPS1 was immunoprecipitated with FLAG antibody conjugated beads and blotted with the phospho-AKT substrate antibody (pAS).

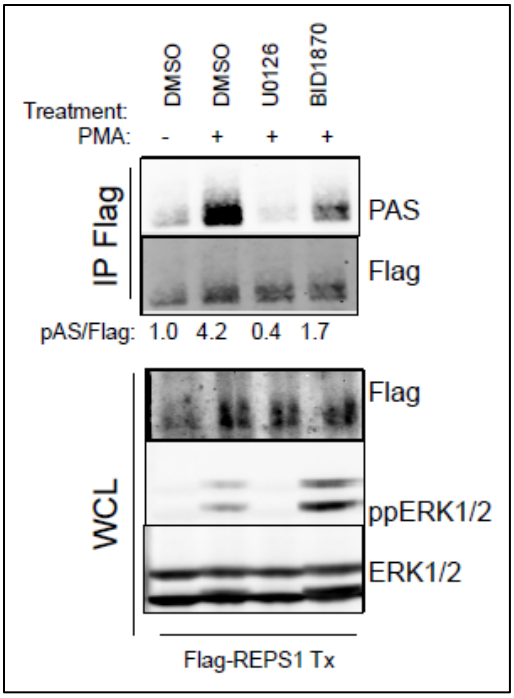

Figure 4.4 REPS1 Ser709 Phosphorylation requires RSK activity. HEK293E cells were transfected with FLAG-REPS1. Cells were stimulated with PMA (100ng/mL) in the presence or absence of MEK and RSK inhibitors, U0126 and BI-D1870. Tx: transfection

Another substrate phosphorylated downstream of ERK D domain was NIPA. NIPA is a constitutively nuclear protein that regulates cyclin B abundance and cell cycle(Bassermann et al., 2007). We identified S407 phosphorylation downstream of ERK D domain signaling. The phosphorylation site lied in the consensus RSK target motif suggesting that RSK may phosphorylate NIPA. To test this, we purified GST tagged NIPA from bacterial cells to use it as a substrate in *in vitro* kinase assay. When HA tagged RSK was immunoprecipitated from PMA stimulated cells, but not from PMA stimulated cells in the presence of U0126, RSK was able to phosphorylate NIPA *in vitro* (Figure 4.5).

We also found identified phosphorylation of MELK Ser415 and Ser409 downstream of ERK2 DEF signaling. MELK is a kinase involved in stem cell maintenance and function and is over-expressed in a variety of cancers (Gray et al., 2005; Nakano et al., 2005). Unfortunately, we did not identify the same phosphopeptides in the ERK2 D domain signaling cells, which prohibits any conclusions about whether MELK is specifically activated by ERK2 DEF signaling. However, it is important to note that MELK has a very high stringency ERK DEF domain on Ser442. Most ERK2 DEF domains are located about 20 amino acids downstream of the ERK phosphorylation site on substrate proteins (Cargnello and Roux, 2011). The presence of the ERK DEF domain 33 and 27 amino acids downstream of the ERK2 phosphorylation sites we identified suggests that MELK may be a *de novo* ERK2 DEF substrate.

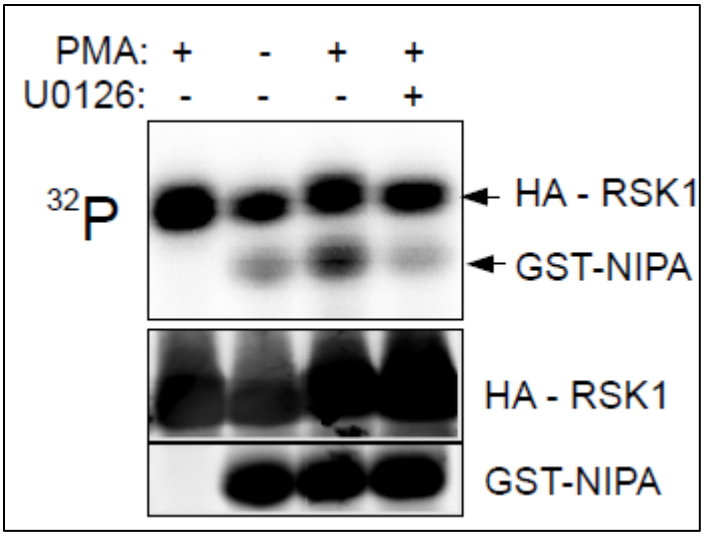

Figure 4.5 RSK1 phosphorylates NIPA *in vitro.* HEK293T cells were transfected with avian HA tagged RSK1 and stimulated with or without PMA in the presence or absence of U0126. RSK1 was immunoprecipitated with HA-antibodies and used as the kinase to phosphorylate bacterially purified GST-NIPA in the presence of radioactively labeled ^{32}P-ATP. Western blots are representative of two independent experiments.

ERK2 D and DEF signaling may differentially regulate distinct crosstalk mechanisms.

Further analysis of the ERK2 D and DEF phosphoproteomes revealed that rpS6 Ser240 phosphorylation, which is carried out exclusively by S6K, was also up-regulated specifically in ERK2 D cells but not ERK2 DEF cells upon EGF stimulation. We hypothesized that upregulation of S6K might reflect increased mTORC1 activity in ERK2 D cells. In support of this idea 4EBP1 Ser65-Ser70 phosphorylation were also enhanced in ERK2 D cells. Increase in the S6K signaling at the 10 minute time point was specific to mTORC1 arm of PI3K signaling as AKT2 and AKT3 hydrophobic motif phosphorylation, which indicate AKT activity, as well as AKT substrate PRAS40 Thr246 phosphorylation were all stimulated to similar extends in both cell lines after 10 minutes. On the other hand, these phosphorylation events were sustained only in ERK2 D signaling but not ERK2 DEF signaling cells 60 minutes following EGF stimulation. Neither IRS1 Ser636 nor RICTOR Ser21 phosphorylation was differentially regulated at the 60 minute time point suggesting that differential regulation of S6K mediated feedback signaling to PI3K could not explain the observed sustained AKT activation in ERK2 D cells (Table 4.2).

Table 4.2 mTORC1 signaling in 10 minute and AKT signaling at 60 minute time points are enhanced in ERK2 D cells as compared to ERK2 DEF cells.

Mutant Time(min) Substrate	ERK2 DEF 10	ERK2 D 10	ERK2 DEF 60	ERK2 D 60
RPS6 Ser240	1.06	5.33	0.62	0.91
EIF4EBP1 Ser70	1.38	2.01	0.84	1.1
EIF4EBP1 Ser65	1.31	2.15	0.93	1.09
AKT2 Ser474	3.17	4.73	1.17	1.83
AKT3 Ser 472	2.76	5.72	1.17	2.3
PRAS40 Thr246	2.05	2.82	0.53	4.59
IRS1 Ser636	N/A	N/A	3.15	2.97
RICTOR Ser21	0.92	0.92	0.95	0.9

ERK2 D signaling also increased MSK2 Ser343-Ser347 phosphorylation, in line with the hypothesis that ERK2 can activate both RSK and MSKs in the context of EGF stimulation (Table 4.3). Surprisingly, we also detected a very high level of induction of RSK1 Ser363 and Ser369 phosphorylation in each cell line suggesting that the ERK1 activity may be sufficient to activate RSK1 in the absence of ERK2 D domain signaling. These phosphorylation events were also present at the 60 minute time point suggesting sustained RSK1 activity. Interestingly, RSK2 phosphorylation on Ser715 was only induced in ERK2 D signaling cells. This is an autophosphorylation site by which RSK phosphorylation induces RSK dissociation from ERK. The finding that RSK2 but not RSK1 phosphorylation is differentially regulated between these two cell lines suggests that ERK1 activity may be sufficient to induce RSK1 but not RSK2 activity. Analysis of SOS1 phosphorylation revealed that this phosphorylation was similarly induced between ERK2 D and ERK2 DEF cell lines. Taken together with the data obtained from BI-D1870 treated cells, these data suggest that SOS1 Ser1134 may be a RSK1 specific targets but this hypothesis should be tested.

Table 4.3 Phosphorylation of MSK2 and RSK2 are enhanced in ERK2 D cells

Mutant	ERK2 DEF	ERK2 D	ERK2 DEF	ERK2 D
Time(min)	10	10	60	60
Substrate				
MSK2 Ser343	1.3	3.73	NA	NA
MSK2 Ser347	1.12	5.05	NA	NA
RSK1 Ser363	7.05	19.26	1.91	2.21
RSK1 Ser369	7.11	19.26	1.91	2.21
RSK2 Ser415	1.36	1.7	0.98	0.97
RSK2 Ser715	1.14	2.3	N/A	N/A
SOS1 Ser1134	15.66	26.84	2.18	3.28

Conclusions and Future Directions

In this chapter we tried to identify targets of ERK2 D and ERK2 DEF signaling pathways that can help us explain the different biological processes regulated downstream of these signaling axis. We found that ERK2 D cells increased AKT3 signaling at the 60 minute and mTORC1-S6K1 signaling at the 10 minute time points. We could not identify differential phosphorylation of any known regulators of AKT signaling that could explain the increased AKT3 phosphorylation in ERK2 D signaling cells. This suggests that there may be a novel crosstalk mechanism between ERK2 D and AKT signaling to be discovered. However, increased S6K1 signaling maybe explained by increased RAPTOR Ser722 phosphorylation, which is known to activate mTORC1 signaling to downstream S6K1 and 4EBP1.

ERK2 D signaling cells proliferate at a much faster rate than ERK2 DEF signaling cells. One hypothesis is ERK2 D cells may be proliferating faster due to parallel activation of mTORC1-S6K1 signaling, which is absent in ERK2 DEF signaling cells. Alternatively, RSK2 activation in ERK2 D cells may drive NIPA phosphorylation leading to more efficient cell cycle. It is important to note that MSK2 phosphorylation on Ser343 and Ser347 were also specifically up-regulated in ERK2 D cells. Despite the evidence that RSK can phosphorylate NIPA *in vitro*, it is also possible that in ERK2 D cells, active MSK2 is phosphorylating NIPA. Given that both MSK2 and NIPA are constitutively nuclear proteins, the hypothesis that MSK2 phosphorylates and regulates NIPA should be tested.

We also found that RSK mediated feedback and crosstalk to SOS1 was intact in both cell lines possibly due to unaltered RSK1 activation in both cell lines. This suggests that differential regulation of RSK mediated feedback may not be sufficient to explain the different biological

processes regulated downstream of ERK2 D and ERK2 DEF signaling axes. On the other hand, RSK2 was specifically phosphorylated downstream of ERK2 D signaling. It is possible that RSK isoforms may be getting differentially regulated by ERK isoforms, however this hypothesis requires more experimentation to be validated. A major caveat of our phospho-proteomics analysis is that direct comparison between different cells lines in terms of fold induction of phospho-peptides may not be accurate. This is because of the fact that except for the EGF starved and stimulated samples, all other samples were processed and analyzed separately for each mutant cell line and each time point suggesting that there may be experimental errors between each data set. Therefore, more accurate comparisons of signaling cascades downstream ERK2 D and ERK2 DEF signaling would require western blot validation or a new phospho-proteomic approach. One such approach is multiplexing where more than two samples can be simultaneously processed, digested and labeled (Dephoure and Gygi, 2012).

Because expression of ERK2 D319N, which is defective in RSK activation, is sufficient to induce EMT, it is possible that RSK activity is dispensable for ERK2 DEF signaling induced EMT. On the contrary, expression of the oncogenic RAS V12 and B-RAF V600E in MCF10A cells require RSK activity for executing an EMT-like transcriptional profile as RSK inhibition blocks both RAS and RAF induced EMT (Doehn et al., 2009; Smolen et al., 2010). Our data suggest in ERK2 D319N cells RSK1 activity may be sufficient to drive event that require RSK activity, yet this hypothesis should also be tested further.

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

Crosstalk and Cell Type Specificity

Despite being thought as linear cascades, over a decade of work has shown that there is intricate crosstalk between growth, stress and metabolic pathways (Mendoza et al., 2011a). In regulation of complex biological processes such as multicellular development, it is not surprising that the organism requires interpretation of environmental signals not only at the organismal but also at the cellular level. Therefore, it is important to note that the extent of crosstalk between each signaling cascade may depend on the developmental stage of the cell, the cell's genetic makeup or the extracellular environment that surrounds the cell. All these factors not only determine how each signaling pathway is wired in different types of cells, but also determine how each signaling cascade will respond to perturbations to the parallel pathways. In our work we focused mostly on breast epithelial cell lines because they have acquired a small number of mutations and this provides a tractable model system. The next stage of these studies would be to understand whether the crosstalk and feedback mechanisms we identified are relevant to different cell lines and if not determine why.

For example, we found that AKT and PIKfyve regulate EGFR degradation in HMECs and MCF10A cells. However, AKT activity was not required for EGFR degradation in MDA-MB-468 cells (not shown). There are four different reasons why AKT activity would not be required for EGFR degradation in MDA-MB468 cells: 1) PIKfyve may be regulated related by kinases, such as SGK, S6K or RSK in this experimental system. 2) EGF induced a very small amount of EGFR degradation. This may be because *EGFR* is amplified at the genomic locus of these cells and highly over-expressed compared to HMECs and MCF10A cells. Therefore, even at very high concentrations of EGF stimulation, the endocytic capacity reaches saturation leading to reduced EGFR degradation. 3) MDA-MB468 cells also do not have functional PTEN. This

may cause a different steady state concentration of each phosphoinositide, which can no longer be regulated by AKT. 4) In our western blot analysis we noticed that PIKfyve from MDA-MB468 cells undergoes a mobility shift, which has previously been shown to be associated with inactivation of PIKfyve (Coronas et al., 2008). It is possible that other signals in MDA-MB468 cells keep PIKfyve in an inactive state, which cannot be activated by AKT. Future work will be required to discriminate between these different possibilities.

One possible method for identifying signaling cross talk in different cell types in a time and dose (either drug or stimulant) dependent manner is through utilization of high through put systems. Multiplexing approach using mass spectrometry is a suitable approach to quantitatively determine proteomic changes in multiple samples. Modifications to the existing technology so that it can quantitatively compare phosphorylation events will certainly enable to us to perform non-biased high content signaling. Combined with other high content analyses such as genomic and transcriptomic profiling, phospho-multiplexing would reveal signaling connections that we were not described before. Because this technology is not currently available we utilized a reductive dimethylation approach to compare, in a pair wise fashion, the effect of EGF stimulation in two different cell lines engineered to express ERK2 D and ERK2 DEF mutants at two different time points following EGF stimulation. Even our initial analyses revealed that there is differential regulation of ERK crosstalk to AKT and mTORC1 pathways induced by a single amino acid mutation in these cells. It is also important to note ERK2 Glu320, which is in the ERK2 DEF binding pocket, is found to be mutated in an oral cancer cell line to Lys (Arvind et al., 2005). Analysis of the genomic mutations in cancer using catalogue of somatic mutations in cancer (COSMIC) (Forbes et al., 2011), revealed that in total ERK2 D319N and ERK2 E320K mutations were detected three times out of 21 ERK2 mutations detected up to date. Taken

together these findings suggest that understanding the signaling downstream of ERK2 docking domains may have clinical relevance to a group of patients.

Crosstalk in Health and Disease

We found that AKT regulation of EGFR degradation can regulate signaling to EGFR signaling to ERK and RSK. EGFR signaling to the PI3K-AKT and ERK-RSK pathways is regulated by endocytosis at multiple levels. Blocking EGFR internalization by expressing EGFR mutants or dominant-negative Dynamin reduces ERK and AKT activation (Goh et al., 2010; Vieira et al., 1996). In addition, the route of EGFR internalization determines signal duration. Clathrin-dependent EGFR endocytosis promotes receptor recycling and sustained AKT activation, whereas non-clathrin dependent EGFR endocytosis promotes transient AKT activation and EGFR degradation (Sigismund et al., 2008). Once internalized, EGFR remains associated with the signaling adapter Grb2 in the early endosomes (Di Guglielmo et al., 1994). EGFR signaling from the endosomes is sufficient to induce DNA synthesis and activates ERK and AKT in HeLa cells and regulates apoptosis during zebra fish development (Pennock and Wang, 2003; Schenck et al., 2008; Zoncu et al., 2009). EGFR signaling to the ERK pathway is also regulated at the late endosomes by the p14-MEK1-MP1 complex (Teis et al., 2006; Teis et al., 2002).

Our finding that AKT regulates EGFR trafficking and degradation suggests AKT can regulate EGFR signaling by regulating EGFR trafficking. AKT inhibition increases phospho-EGFR Tyr1068 and phospho-ERK levels in MCF10A cells. The AKT1 isoform negatively regulates ERK signaling in these cells via an unknown mechanism (Irie et al., 2005). We show

that the AKT inhibitor-induced up-regulation of ERK activation requires active EGFR, consistent with AKT-facilitating EGFR degradation as a mechanism to reduce ERK signaling.

Trafficking and degradation are important components of oncogenic RTK signaling. Mutant EGFRs that drive lung cancer proliferation degrade slower than wild type EGFRs, associate less with the ubiquitination machinery, and co-localize more with the markers of the recycling endosomes (Chung et al., 2009; Shtiegman et al., 2007). Constitutively active c-Met mutants require receptor endocytosis to promote migration and in vivo transformation (Joffre et al., 2011). Our data suggest that therapeutic targeting of AKT in settings where RTKs are hyperactive should be performed with caution, due to AKT's ability to facilitate EGFR trafficking and degradation and inhibit ERK signaling. Thus, inhibiting AKT signaling in these cells may actually promote tumorigenesis by reducing the cell's ability to clear surface receptors and inducing continued RTK signaling from the endosomes. Determining the contribution of the AKT-mediated feedback to multiple RTKs through activation of PIKfyve in the context of various cancer cell types will enhance our understanding of RTK biology and the intricate downstream signaling networks.

In addition to cancer, AKT crosstalk to ERK may also be relevant to diabetes where AKT is chronically inhibited. In the context of type 2 diabetes, the adipose tissue is unable to stimulate AKT activity in response to insulin. Reduction in AKT activity leads to inefficient glucose uptake by the cells because AKT mediated GLUT4 translocation to the cell membrane is disrupted. One mechanism that contributes to the cells' inability to activate AKT in response to insulin is due to hyperphosphorylation of IRS proteins on serine residues, which prevent signaling from insulin receptor to PI3K. Both S6K and ERK have been shown to phosphorylate IRS proteins. It is possible that when AKT is inhibited, ERK is hyperactived through

accumulation of cellular RTKs because of disrupted RTK degradation. Hyperactive ERK may then increase IRS phosphorylation to inhibit Insulin receptor signaling to PI3K. This would then cause even further inhibition of AKT leading to more severe defects in glucose uptake by the cells. Therefore, in type two diabetes, pharmacological inhibition of ERK may reduce IRS phosphorylation leading to reactivation of insulin-PI3K-AKT signaling axis and therefore allowing for proper glucose uptake and alleviated disease. However, these hypotheses should be tested further.

Our discovery of RSK mediated MEKK3 inhibition adds another level of complexity to the crosstalk between growth, stress and metabolic pathways. MEK1-2, PI3K, AKT, mTORC1 and EGFR inhibitors are all in clinical trials for treatment of cancers that show activation of these pathways. However, we currently do not understand whether these inhibitors regulate different biological processes in tumor and normal cell lines. Several lines of experimental and clinical evidence show that MEK1-2 inhibitors reduce survival and proliferation of tumor cells that are addicted to ERK signaling. However, the long term effects drug treatments, such as deregulation of cytokine production by either the stroma or the tumor cells due to relief of RSK mediated feedback to MEKK3 needs to be tested. Given that the immune system has critical functions in tumor maintenance, metastasis and clearance, it is necessary to investigate the role of RSK crosstalk to p38, ERK5 and possibly to NFkB signaling pathways through inhibition of MEKK3 in the context of cancer biology.

Finally, another context that the cytokine production plays an important role is in metabolic syndromes where chronic inflammation contributes to the severity of the disease. If RSK regulation of MEKK3 alters cytokine production, it will be important understand what role

RSK activity or lack thereof plays in pathogenesis of metabolic syndromes such as obesity and type 2 diabetes.

Chapter 6

MATERIALS AND METHODS

Cell culture and transfections

Human Mammary Epithelial cells were immortalized, maintained and infected pBabeNeo EGFR retrovirus as previously described (Mendoza et al., 2011b). For knockdown experiments cells were plated at a density of 200-250 thousand cells per 6 cm dishes 24 hours before transfection with Allstars non-targeting control siRNA (Qiagen) or siRNAs targeting AKT1, RAB11a, and ArPIKfyve, PIKfyve (Qiagen) or SAC3 (Dharmacon) using Lipofectamine RNAimax reagent (Invitrogen). MCF10A cells were cultured as previously described (Irie et al., 2005). HEK 293T and 293E cells were cultured in DMEM containing 10% FBS. Calcium phosphate transfections in HEK293 cells were done as previously described (Mendoza et al., 2011b).

List of siRNA target sequences

AKT1-1: aatcacaccacctgaccaaga

AKT1-2: cacgcttggtcccgaggccaa

RAB11-1: aagagtaatctcctgtctcga

RAB11-2: cgaaatgagtttaatctggaa

SAC3-1: gaacaaagatgggacagaa

SAC3-2: gaagttatctgtgctgtga

SAC3-3: gtagtgagccttatatgaa

SAC3-4: tctataaggtcgaagatac

PIKFYVE: cagagatgagtatgcgctgta

ArPIKfyve: ccggagggttgtcctctgaca

Antibodies and reagents

EGFR, pEGFR (Tyr1068), pAKT (Ser473), pS6K (Thr389), pPRAS40(Thr246), PRAS40, Ubiquitin, MEKK3, pAKT substrate antibody (110B7E) antibodies were from Cell Signaling Technologies. Antibodies were from the following companies GAPDH (Ambion), FLAG tag, ppERK1/2 (Sigma), S-tag (Novagen), S6K1, ERK and HA antibodies were made in house, EEA-1 (Santa Cruz Biotechnologies), Rab11 (BD biosciences). EGFR antibody used for immuno-precipitation, immuno-fluorescence and flow cytometry (Calbiochem), ArPIKfyve and PIKfyve (Abcam). Secondary Alexa-Fluor 488, 674 and 568 antibodies and Alexa-488 labeled EGF were purchased from Molecular Probes, Invitrogen. Secondary antibodies for western blotting was from Li-COR. Gefitinib (2μM final), PI103 (5μM final) and U0126 (20 μM Final), BI-D1870 (10μM final) and SL0-101(100μM final) was from Selleck Biochemical. AKTVIII (10μM final) and YM201636 (200nM final) were from Calbiochem. All inhibitors were dissolved in dimethyl sulfoxide (DMSO) with the exception of SL0-101 which was dissolved in ethanol. All inhibitors were stored as aliquots at -20^{o}C with the exception of AKTVIII which was stored at -80^{o}C. DMSO, N.E.M (N-ethylamimide), Cyclohexmide and Chloroquine was from Sigma-Aldrich. EGF, Insulin was from Pepro-Tech. AKT catalytic inhibitor A-443656 was a kind gift from Dr. Nathaniel Gray.

Cell Lysis, immuno-precipitation and western-blotting

Cell lysis was performed as previously described in the presence of protease and phosphatase inhibitors (Mendoza et al., 2011b). For ubiquitination experiments 10μM NEM was

added to the lysis buffer. EGFR was immuno-precipitated with EGFR antibody or mouse IgG and with a 1:1 mixture of Protein A (Sigma-Aldrich) and Protein G Sephrose Beads (GE Health Care) at 4^{o}C for 90 minutes. FLAG PIKfyve was immuno-precipitated using agarose conjugated FLAG antibodies (Sigma, Aldrich). HA-ArPIKfyve and HA-RSK were immunoprecipitated with anti HA antibody with Protein A. Beads were spun at 5000rpm for 1 minute and the flow-through was discarded. Beads were washed three times with lysis buffer and boiled with SDS loading buffer at 100 ^{0}C for 6 minutes. Western blotting and quantification using the Odyssey Li-COR imaging system was carried out as previously described (Mendoza et al., 2010).

EGFR degradation assays

EGFR degradation assays were carried out by depriving cells of growth factors and or serum overnight and stimulating them with 100ng/mL EGF unless otherwise stated. EGFR protein abundance at each time point before EGF stimulation (EGFR(0min)) and after EGF stimulation (EGFR(t) where t = 30min, 60min…) was measured by western blotting and quantification of the integrated intensity of EGFR bands with the Odyssey software (Li-COR). Percent undegraded EGFR was determined by calculated by the formula: EGFR(t)/EGFR(0min).

Immuno fluorescence and image analysis

Cells were plated on HCl treated coverslips as previously described (Mendoza et al., 2011b) at a density of 50,000 cells per well in 6-well plates, 48 hours prior to assay. Cells were deprived of growth factors 24 hours prior to staining and treated with inhibitors as indicated. Cells were fixed with 3.7% Formaldehyde and stained overnight with primary antibody for EEA.1 (1:100) and EGFR (1:100). Images were taken using Nikon Ti inverted microscope with Yokagawa CSU-10 spinning disk confocal microscope at 60X magnification. Images were taken

from the focal plane of the nucleus or as Z-stacks at a 0.3μm step size. Percent co-localization values were calculated using the measure co-localization function of the MetaMorph® software. Pixels with intensity values above a fixed threshold from each channel were used for calculations. Statistical analysis of the percent co-localization values was carried out using bootstrapping permutation test (Efron and Tibshirani, 1993). Box plots were generated using MATLAB ® (R2012a, Natick, MA). For measurement of integrated intensities of EGF and EGFR in EEA.1 positive early endosomes, we created early endosomes from EEA.1 pixels that are above the local background and form a continuous shape with a fixed minimum and maximum size. These endosomes were transferred over to the images that contain the EGF/EGFR signal and the EGF/EGFR signal only within these regions was calculated as integrated intensities after a constant background subtraction. Integrated intensities from every other Z-plane were pooled for an entire field. Integrated EGF-EGFR intensities in DMSO and AKTVIII treated cells were compared using Kolmogorov-Smirnov analysis for non-normal distributions. Cumulative distribution function plots were generated using MATLAB ® .

EGFR recycling assay

EGFR recycling was assessed as previously described in (Sigismund et al., 2008) with the following changes. 3 plates of cells were pulsed with 100ng/mL EGF or TGFα for 15 minutes at 37^{o}C to allow for internalization and 1 plate was left un-stimulated (Total Surface EGFR). Internalization was stopped by moving the cell to 4^{o}C. Excess EGF on cell surface was washed away with an acid treatment (150mM NaCl, 50mM Glycine at pH 3.0) for 3 minutes at 4^{o}C and washed with PBS three times. One plate was left at 4 ^{o}C (Pulse). Two plates were moved back to 37^{o}C to allow for recycling for 10 or 20 minutes (Chase). Following the chase period, cells were moved back to 4^{o}C, fixed with 1% Formaldehyde in PBS, washed once with

PBS and twice with STE (10mM Tris-Cl, pH7.5, 10mM NaCl, 1mM EDTA) and collected. Cell suspension was blocked with 3% BSA in PBS at 4^{o}C for 30 minutes and labeled with EGFR antibody (1:100) at 4^{o}C for one hour in 3% BSA and with Alexa-488 donkey anti mouse antibody for 30 minutes at 4^{o}C. Using flow-cytometry 10,000 cells were evaluated for surface EGFR staining. Percent recycling at either 10 or 20 minute time points was calculated using the formula ((Chase – Pulse) /(Total Surface EGFR – Pulse)) * 100 from median values of each treatment group (Figure 6.1).

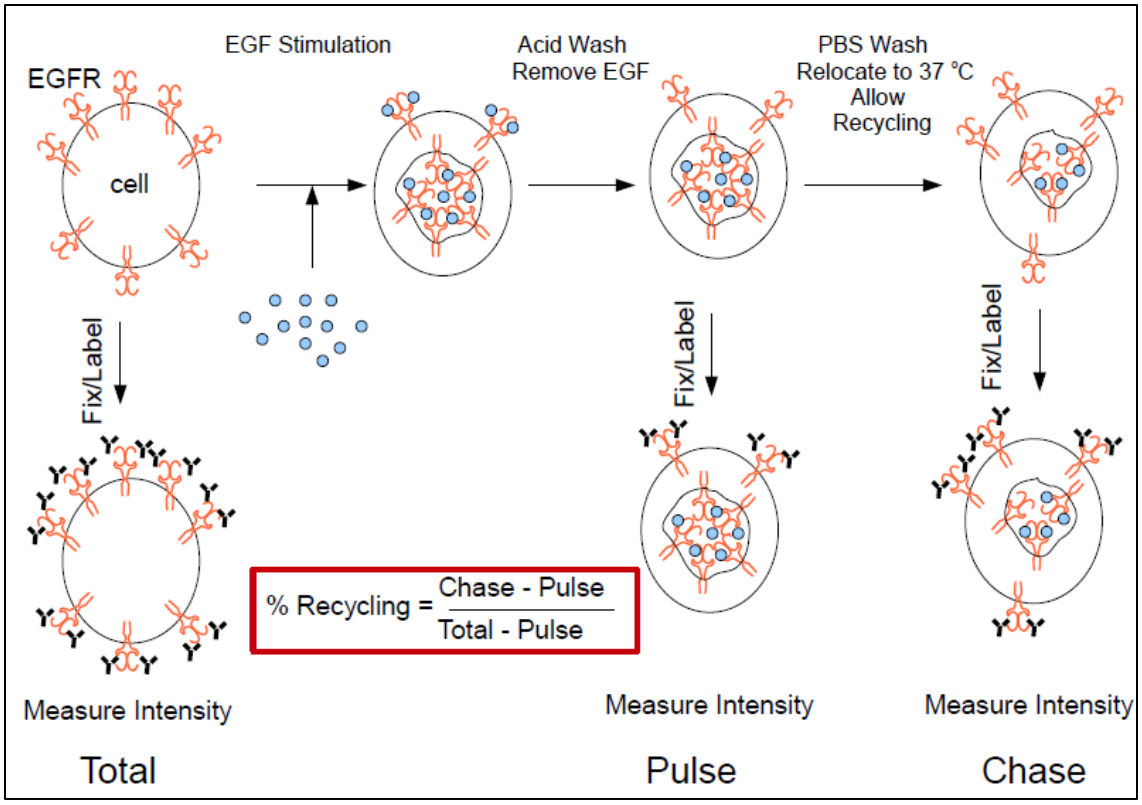

Figure 6.1 EGFR recycling assay

Phosphoinositide phosphatase assays.

FLAG-PIKfyve, S-SAC3 and HA-ArPIKfyve complex was expressed in HEK293T cells. The complex was immunoprecipitated with anti-HA antibodies (30 minutes at 4^{o}C) and Protein A beads (1 hour at 4^{o}C). Immunoprecipitates were washed twice with lysis buffer and twice with phosphatase buffer (50mM Tris-Cl pH7.4, 1mM MgCl2 , 1mM Dithiothreitol). Beads were then resuspend with 35uL of reaction mixture (31uL of phosphatase buffer and 4uL of di-C8 PI(3,5)P2 (Echelon-inc Biosciences). Reactions were carried out at 37 C degrees for 15 minutes for WT SAC3 and for 60 minutes for D488A mutant. 25uL of the reaction was mixed with malachite green assay buffer (Echelon-inc Biosciences). Amount of free phosphate liberated from PI(3,5)P_2 via SAC3 activity in each reaction was read at 660nm along with free phosphate standards and calculated as instructed by the manufacturer.

Phosphoinositide kinase assay

PI3P (Avanti Polar Lipids) vesicles was generated by sonication for 10 minutes in HEPES (30mM pH 7.4) / EGTA (1mM) buffer. FLAG-PIKfyve, S-SAC3 and HA-ArPIKfyve complex expressed in 293T cells was immunoprecipitated as above, washed twice with lysis buffer and twice with TNE buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.5mM EGTA). After the first wash 1/11th of the imunnoprecipated beads were used for western-blotting to ensure equal loading, the rest was used in the phosphoinositide kinase assay. After second wash the beads were resuspended in 65µL TNE buffer, incubated with PI3P vesicles for 5 minutes on ice and incubated with 10 µL ATP reaction mix (6.5 mM HEPES pH 7.4, 50µM ATP, 2.5mM $MnCl_2$, 10mM $MgCl_2$, 5mM β-glycerophosphate, 12.5µCi ^{32}P-ATP final) for 15 minutes at room temperature. Reaction was stopped by adding 4N HCl and phosphoinositides were extracted

using 1:1 Methanol:Chloroform. Phosphoinositides were spotted on Silica TLC (Milipore) and separated using 65:35 2M Acetic acid:n-proponal. Membrane was dried and exposed to Phospho-imager screen (Bio-Rad) and the radioactively labeled PI(3,5)P2 spots' volumes were quantified using Quantity One® software (Bio-Rad).

Bacterial Protein Purification

GST-NIPA and GST-MKK3 were purified from bacterial cells as previously described (Mendoza et al., 2010). Bacteria were transformed with the pGEX-2T GST-MKK3 or GST-NIPA plasmids and plated antibiotic containing LB Agar plates containing 100µg/mL carbenicillin. After overnight growth, single colonies were picked and grown overnight in 10mL of LB media with 100µg/mL carbenicillin at 37^{o}C with constant shaking. The starter cultures were then transferred into 1L of 2xYT media (BD biosciences) with 100µg/mL carbenicillin. OD600 was monitored and bacterial protein expression was induced with 0.2mM Dioxane-free isopropyl-beta-d-thiogalactopyranoside (IPTG) final when OD600 reached 0.8. Induction was continued overnight at 16^{o}C. The bacterial pellets were homogenized in lysis buffer using a dounce homogenizer. For GST-NIPA purification the lysis buffer was 1 × PBS, pH 7.4 with 50 mM EDTA, pH 8. For GST-MKK3 the lysis buffer was 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM MgCl2 and 1% NP-40. The resulting homogenates were passed through a pre-chilled microfluidizer at 20,000 PSI, twice. The lysates were spun at 45.000rpm for 30min in an ultracentrifuge. GST proteins in the supernatant were immune-precipitated with Glutathione Sepharose 4B beads (GE Healthcare) at 4^{o}C for 1h. GST beads were washed in a chromatography column using gravity. GST proteins were eluted with lysis buffer with 10mM glutathione pH 7.5 and snap frozen in liquid nitrogen.

Protein kinase assays

Protein kinase assays using RSK as the kinase and GST-NIPA as the substrate was similar to described before (Mendoza et al., 2010). HA-avRSK1 was immunoprecipitated from cells. The immune-precipitates were washed twice in lysis buffer and twice in kinase buffer (25 mM Tris base (pH 7.4), 1 mM DTT, 10 mM MgCl2, 2.5 mM b-glycerophosphate). Kinase assay was performed in kinase buffer with 125μM ATP and 5μCi ^{32}P-ATP for 15 minutes at 30 degrees.

Protein kinase assays using MEKK3 as the kinase and GST-MKK3 was performed by lysing the HA-MEKK3 expressing cells using lysis buffer (20 mM Tris-Cl at pH 7.4, 1% NP-40, 135 mM NaCl, 10% glycerol, 0.1 mM EDTA and 0.1mM EGTA). HA-MEKK3 immunoprecipitates were washed twice in lysis buffer and twice in kinase buffer. MKK3 and MEKK3 was incubated in kinase buffer with 1mM DTT, 10mM beta-glycerophosphate 0.1mM sodium ortho vanadate, 125 μM ATP, 5μCi ^{32}P-ATP for 12 minutes at 30 degrees.

The kinase reactions were stopped by adding 5X SDS loading buffer and boiling samples at 80^{o}C. The kinase reactions were resolved by SDS-PAGE and proteins were transferred onto membrane. Incorporated radioactivity onto MEKK3, MKK3, RSK and NIPA was calculated by exposing the membrane to Phosphor-Imager (GE Healthcare) and quantifying radioactivity using Quantity One image software.

Measurement phosphoinositides in cells

HMECs are plated at 1 million cells per 10 cm plate and phosphoinositides were radioactively labeled by growing cells with inositol free DMEM/F-12 media (U.S. Biologicals) supplemented 10μCi/mL 3H-myo-inositol (American Radiolabeled Chemicals) for 48 hours

with. Cells were then starved for growth factors by incubation in inositol free DMEM/F12 media without EGF and Insulin for 1 hour. Inhibitors were added at the last 30 minutes of starvation. Cells were then stimulated with EGF (100ng/mL) for 30 min. Phosphoinositides were then extracted, deactylated and seperated by HPLC as described previously (Sarkes and Rameh, 2010).

Mass spectrometry analysis

Mass spectrometry was performed on MCF10A cells where endogenous ERK2 was knocked down and ERK2 D (Y261A) or ERK2 DEF (D319N) was re-expressed. Cells were deprived of serum, EGF and insulin overnight and either stimulated with 10ng/mL EGF for 10 minutes or 60 minutes or left unstimulated. Cells were lysed with 8M urea and sonicated. The supernatants were normalized for protein concentration. 5mg of protein lysate from each cell line and each treatment was denatured by adding DTT and heating to 56^{o}C. Unreacted DTT was quenched, samples were trypsin digested. Resulting peptides were desalted using tC18 SepPak solid-phase extraction cartridges and labeled using reductive dimethylation (Khidekel et al., 2007). Following labeling peptides were run on strong cation exchange chromatography as previously described (Villen and Gygi, 2008). Phospho-peptides were further enriched by titanium dioxide enrichment and were run on LC-MS/MS. The resulting heavy to light ratios were generated as previously described (Villen and Gygi, 2008).

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